Mass Spectrometric Characterization of Circulating Covalent Protein Adducts Derived from Epoxide Metabolites of Carbamazepine in Patients

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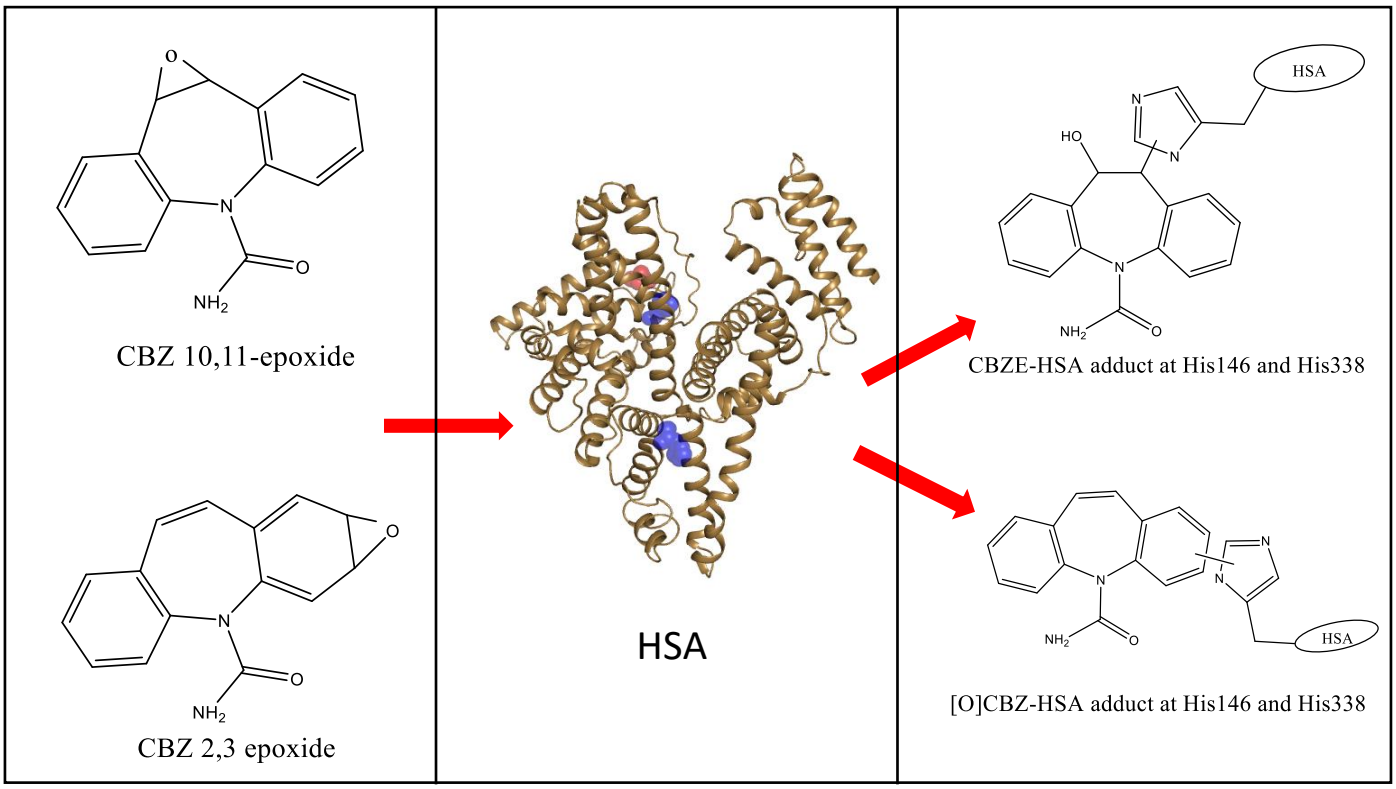
TOC Graphic


CBZ 10,11-epoxide
CBZ 2,3 epoxide
HSA
CBZE-HSA adduct at His146 and His338
[O]CBZ-HSA adduct at His146 and His338

ABSTRACT: Carbamazepine (CBZ) is an effective antiepileptic drug that has been associated with hypersensitivity reactions. The pathogenesis of those reactions is incompletely understood but is postulated to involve a complex interplay between the drug's metabolism, genetic variation in human leukocyte antigens and adverse activation of the immune system. Multiple T-cell activation mechanisms have been hypothesised, including activation by drug-peptide conjugates derived from proteins haptenated by reactive metabolites. However, definitive evidence of the drug-protein adducts in patients has been lacking. In this study, mass spectrometry was used to characterize protein modifications by microsomally generated metabolites of CBZ and in patients taking CBZ therapy. CBZ 10,11-epoxide (CBZE), a major electrophilic plasma metabolite of CBZ, formed adducts with glutathione-*S*-transferase pi (GSTP; Cys47) and human serum albumin (HSA; His146 and His338, but not Cys34) in vitro, via notably divergent side-chain selectivity. Both proteins were adducted at the same residues by undefined monoxygenated metabolites ([O]CBZ) when they were incubated with human liver microsomes, NADPH and CBZ. There was also evidence for formation of a CBZ adduct at His146 and His338 of HSA derived via dehydration from an intermediate arene oxide adduct. Glutathione trapping of reactive metabolites confirmed microsomal production of CBZE, and indicated simultaneous production of arene oxides. In 15 patients prescribed CBZ therapy, [O]CBZ-modified HSA (His146) was detected in all the subjects. The relative amount of adduct was moderately positively correlated with plasma concentrations of CBZ ($r^2 = 0.44$, $p = 0.002$) and CBZE ($r^2 = 0.35$, $p = 0.006$). Our results have provided the first chemical evidence for microsomal production of [O]CBZ species that are able to escape the microsomal domain to react covalently with soluble proteins. This study has also demonstrated the presence of circulating [O]CBZ-modified HSA in patients without hypersensitivity reactions who were receiving standard CBZ therapy. The implications of those circulating adducts for susceptibility to CBZ hypersensitivity merits further immunological investigation in hypersensitive patients.

INTRODUCTION

Carbamazepine (CBZ) is an effective treatment for epilepsy but up to 10% of patients experience a hypersensitivity reaction.[1] Those reactions can range from mild to severe life-threatening conditions.[2] The reactions principally involve the skin but can also include other organs such as the liver and kidneys.[2] The pathophysiology of hypersensitivity to CBZ is incompletely understood but has been hypothesised to involve a complex interplay between the drug's metabolism, genetic variation in human leukocyte antigens (HLA) and activation of the immune system.

The clinical features of CBZ hypersensitivity in conjunction with the identification of CBZ-specific T cells from patients with a history of hypersensitivity to CBZ confirms an immunological aetiology.[3] Increased susceptibility to CBZ-induced reactions has been observed in patients of South-East Asian origin who possess *HLA-B*15:02*.[4] HLA alleles encode the major histocompatibility complex (MHC), which presents protein-derived peptides to T cells as part of the adaptive immune response. MHC class I molecules are encoded by *HLA-A*, *HLA-B* and *HLA-C* whilst MHC class II molecules are encoded by *HLA-DR*, *HLA-DQ* and *HLA-DP*.[5] In other ethnicities, *HLA-A*31:01* has been significantly associated with increased risk for multiple phenotypes of CBZ-induced cutaneous hypersensitivity.[6, 7] Functional studies investigating the pathomechanisms of CBZ hypersensitivity using T cell clones generated from hypersensitive patients support direct activation of T cells by CBZ, via ligand binding, i.e. a pharmacological interaction (PI) mechanism.[3, 8, 9] Further studies have claimed that *HLA-B*15:02* on antigen presenting cells presents CBZ directly to specific T-cell receptors (TCR), thereby activating a cytotoxic immune response.[10, 11] Structural modelling of the *HLA-B*15:02*/peptide/CBZ/TCR complex revealed the CBZ molecule to be located at the interface between *HLA-B*15:02*/peptide and TCR with CBZ having higher affinity for TCR over *HLA-B*15:02*/peptide.[12] The authors postulate two hypotheses: (i) the presence of specific TCR subtypes increases interaction between TCR/CBZ and *HLA-B*15:02*/peptide complex leading to activation of the immune system; and (ii) binding of CBZ to TCR shifts the receptor's specificity and thereby the CBZ-modified TCR is able to recognise self-peptides presented by *HLA-B*15:02*, causing an autoimmune reaction. Finally, the major electrophilic metabolite of CBZ, carbamazepine 10,11-epoxide (CBZE), is also

able to bind *HLA-B*15:02* in vitro, raising the possibility that CBZE may be the responsible immunogen in CBZ hypersensitivity.[13]

The metabolism of CBZ in humans is complex, yielding more than 30 metabolites.[14] Bioactivation of CBZ by P450 in human liver microsomes was revealed by the formation of protein-reactive metabolites.[15, 16] The reactive species were not identified but from their deactivation by glutathione (GSH) were evidently soft electrophiles.[15] It has been hypothesised that multiple reactive intermediates of CBZ, such as arene oxides,[15, 17, 18] 9-acridine carboxaldehyde,[19] iminostilbene,[20, 21] an *o*-quinone[22] and CBZE,[21] are responsible for the protein adduct formation (Scheme 1). Importantly, CBZE, an abundant[23] and pharmacologically active plasma metabolite[24] formerly believed to be chemically stable, has been demonstrated to be reactive through the spontaneous formation of two isomeric CBZE-glutathione adducts in vitro (CBZE-SG1 and CBZE-SG2) and its covalent binding to human liver microsomes.[25] However, with the exceptions of CYP1A2[26] and CYP3A4[27], none of the microsomal proteins that form covalent adducts with the reactive metabolites of CBZ has been identified. Molecular modelling alone has suggested Cys239 of CYP3A4 could be a haptenation site,[27] creating a thioether antigen to which the immune response may be directed.[28] That deduction, the reaction of CBZE with GSH,[25] and the trapping of CBZ's reactive metabolites by GSH in microsomal incubations[29] predict modification of proteins at cysteine residues. The inferred structures of the multiple GSH adducts produced from CBZ by human liver microsomes included thioether derivatives of one or more arene oxides, namely products of nucleophilic conjugate addition, as well as the two adducts of CBZE.[29] Nonetheless CBZE-SG1 and CBZE-SG2 were the most abundant adducts formed from CBZ. Consequently, it was hypothesised that CBZE will be the principal protein-adducting metabolite of CBZ.

Although much of the experimental evidence suggests a direct, i.e. PI, mechanism for CBZ hypersensitivity, in vitro systems are only able to test the activity of stable and weakly electrophilic candidate antigens; highly reactive metabolic intermediate that haptenate proteins cannot be tested equally. It is possible that CBZ is able to activate the immune system via multiple mechanisms, including both PI and hapten effects,

depending on the metabolic capacity and HLA status of each patient.[30] The existence of multiple immune activation mechanisms may explain the variation in clinical presentation of CBZ hypersensitivity.

The aims of this study were to characterize the structures of the protein conjugates formed by CBZ's reactive metabolites and hence derive the metabolic pathways of their formation, using HSA and glutathione *S*-transferase pi (GSTP) as model proteins in vitro. GSTP is a proven biochemical reagent for trapping highly reactive small-molecule thiophilic electrophiles,[31-34] its three nucleophilic cysteines (Cys14, 47 and 101) exhibiting markedly disparate adduction capabilities.[33] HSA has a much more varied set of adductable nucleophilic side-chains.[35-38] We have previously isolated and identified conjugates of circulating HSA with intrinsically reactive drugs,[35, 39, 40] circulating reactive drug metabolites[36, 41] and metabolic intermediates that might be confined to intracellular locations.[36, 37] CBZE is an electrophilic species and a major plasma metabolite in patients,[23] whereas the arene oxides, by analogy with the instability of bromobenzene-3,4-oxide,[42] are likely to be fleeting entities in blood and to react only with cellular proteins. CBZE might haptenate HSA in the bloodstream and the protein within hepatocytes that is destined for export. Substantial intravascular haptenation by CBZE could manifest itself as an association between the relative abundance of [O]CBZ-HSA adducts and plasma concentrations of CBZE. HSA isolated from patients exposed to CBZ therapy was analysed to determine if adducts similar to those generated in incubations with human liver microsomes could be detected. It is important to characterise the haptenation by CBZ in patients. Conjugates of reactive monoxygenated metabolites ([O]CBZ) on HSA and other proteins have the potential to act as antigens that trigger the immunological signals responsible for CBZ's hypersensitivity.

EXPERIMENTAL PROCEDURES

Chemicals. HSA (97-99% pure) was purchased from Sigma-Aldrich (Poole, Dorset, United Kingdom), sequencing-grade modified trypsin from Promega (Southampton, United Kingdom), liquid chromatography-mass spectrometry (LC-MS) grade solvents from Fisher Scientific UK Ltd. (Loughborough, Leicestershire, United Kingdom), and all other standard reagents from Sigma-Aldrich. CBZ and CBZE were purchased from Sigma-Aldrich. Authentic standards of 2-methylcarbamazepine (2-Me-

CBZ; internal standard), carbamazepine-10,11-trans-dihydrodiol (CBZ-DHD), 2-hydroxycarbamazepine (2-OH-CBZ) and 3-hydroxycarbamazepine (3-OH-CBZ) were purchased from NewChem Technologies (Durham, United Kingdom). The K541 tryptic peptide of HSA (^{535}ATKEQLK^{541}) was synthesised by standard solid-phase chemistry and characterized by LC-MS/MS (Figure S1).[43]

Microsomes and Supersomes. Human liver microsome preparations (UltraPool human liver microsomes 150; a pool from 150 mixed gender donors) were obtained from BD Gentest Corp. (Oxford, United Kingdom). Microsomes (Supersomes) from baclovirus-infected insect cells expressing human P450 enzymes (3A4, 2B6, and 2E1) were purchased from BD Gentest Corp. All recombinant enzymes were co-expressed with human NADPH-cytochrome P450 reductase and human cytochrome b_5.

Patients and Volunteers. Single plasma samples were obtained from epilepsy patients attending the Walton Centre NHS Foundation Trust, Liverpool. Patient details are listed in Table S1 of the Supporting Information. Ethical approval was obtained from the National Research Ethics Service Committee for North West – Haydock (ethics reference: 13/NW/0503). Blood samples were collected from fifteen patients who had received a stable dose of CBZ for at least six months. The timings of the blood samples relative to the last dose of CBZ were recorded. The blood (5 mL) was collected into lithium heparin-containing tubes and was centrifuged at 1500 *g* and 4^{o}C for 10 min to obtain plasma. Plasma samples were stored in aliquots (0.1 mL and 0.3 mL) at -80^{o}C until they were taken for assays of CBZ and its metabolites or the isolation of HSA.

Synthesis of the GSH Conjugates of CBZE and the Trapping of Microsomally Generated Metabolites of CBZ. The two isomeric GSH conjugates of CBZE (CBZE-SG1 and CBZE-SG2) were prepared as authentic metabolite standards by incubating synthetic CBZE (80 μM) with GSH (5 mM) in phosphate buffer (0.1 M, pH 7.4; final volume 0.25 mL) at 37^{o}C for 2 h. The reaction mixture was analysed without further treatment by LC-MS/MS. Human liver microsomes and Supersomes incubations were performed by incubating CBZ (100 μM) with either human liver microsomes (protein concentration, 2 mg/mL) or Supersomes (P450 concentration, 20 nM), NADPH (2 mM), $MgCl_2$ (5 mM) and GSH (2 mM)

for 2 h at 37ºC in phosphate buffer (final volume, 0.2 mL). The reactions were started by addition of NADPH. Protein concentrations in this and all other experiments were determined by the Bradford assay (Sigma-Aldrich). The enzymatic reaction was terminated by addition of two volumes of acetonitrile to the reaction mixture. The mixtures were vortexed, and centrifuged at 12,000 *g* for 5 min at 4ºC. The supernatants were collected, and evaporated to dryness under nitrogen. These residues were reconstituted in 0.1% aqueous formic acid (0.5 mL) before analysis by LC-MS/MS as described below.

Analysis of Oxygenated CBZ Metabolites Produced by Microsomes and Recombinant P450 Enzymes and Characterization of GSH Adducts of CBZ. Reconstituted supernatants from the incubations of CBZ with either human liver microsomes or Supersomes were analysed by LC-MS. Aliquots (5-20 μL) were injected onto a Zorbax Eclipse XDB-C8 column (150 × 4.6 mm, 5μm; Agilent Technologies, Santa Clara, CA) fitted with a Zorbax C18 Reliance pre-column and connected to a PerkinElmer series 200 HPLC system (PerkinElmer, Norwalk, CT). Analytes were eluted at room temperature with a gradient program of acetonitrile (15% to 33% over 12 min; 33% to 50% over 0.5 min; 50% for 5.5 min; 50% to 15% over 0.1 min; 15% for 4 min) in ammonium formate (2 mM, pH 3.8) at 1.0 mL/min. A different eluent was required for analysis of the GSH adducts of CBZ and CBZE, namely a gradient of acetonitrile (5% to 30% over 20 min; 30% to 60% over 0.5 min; 60% for 4.5 min; 60% to 5% over 0.1 min; 5% for 3.9 min) in 0.05% (v/v) formic acid at 1.0 mL/min. That eluent also resolved the CBZ phenols and CBZE. The split-flow to the mass spectrometer was approximately 150 μL/min. Metabolites were detected in positive-ion mode with a 4000 Qtrap instrument (Sciex, Warrington, United Kingdom) that was operated under the following conditions: source temperature, 450 ºC; ionspray voltage, 4,500 V; desolvation potential, 25 V; Gas-1 and Gas-2 settings, 50; scanning, m/z 100-1,000 over 5 s. Instrument management and data processing were accomplished through Analyst 1.5.1 software (Sciex). Analytes were identified by chromatographic and mass spectrometric comparisons with authentic standards. Multiple reaction monitoring (MRM) transitions for CBZ, its oxygenated metabolites and the GSH adducts are specified in Table S2.

Modification of His-Tagged GSTP by Synthetic CBZE and Microsomally Generated Monoxygenated Metabolites of CBZ. His-tagged human GSTP was expressed in *Escherichia coli* as described previously.[44] Synthetic CBZE was freshly dissolved in methanol (50 mM) and incubated with nickel bead-captured GSTP (130 μM), prepared as described by Jenkins et al.,[31] in phosphate buffer (0.1 M, pH 7.4) at 37°C for 6 h. The molar ratio of CBZE to protein was 10:1. Reactive metabolites were generated from CBZ (1 mM) in co-incubations of nickel bead-captured GSTP (40 μM) with either human liver microsomes (2 mg/mL) or Supersomes (CYP3A4, CYP2B6 or CYP2E1, 20 nM), $MgCl_2$ (5 mM), and phosphate buffer, for 6 h at 37°C. The reactions were started by addition of NADPH (final concentration, 1 mM). The final volume was 0.4 mL. After the incubation period, a 0.2 mL aliquot was removed from the reaction mixture, taking care not to remove any nickel beads, and stored at 4 °C for analysis of CBZ metabolite formation (see below). The nickel beads were recovered from the remaining reaction mixture using magnetic separation and washed five times with 1 mL phosphate buffer. Ammonium bicarbonate solution (50 μL of 50 mM, pH 7.0) was added to the beads and the protein subjected to on-bead tryptic digestion (1 μg of trypsin per 200 μg of protein) at 37°C for 16 h. The digest was processed for analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described below.

Concentration-Dependent Modification of HSA by CBZE In Vitro. CBZE freshly dissolved in methanol (50 mM) was incubated with HSA (1 mM, 40 μL) in phosphate buffer at 37°C for 16 h. The molar ratios of CBZE to protein were 0.1:1, 1:1 and 5:1. The final concentration of methanol ranged between 0.1% v/v to 5% v/v. Protein was precipitated twice with nine volumes of ice-cold methanol to remove noncovalently bound CBZE, resuspended in 50 μL of phosphate buffer, and then reduced with 10 mM dithiothreitol (15 min) and alkylated with 50 mM iodoacetamide (15 min) at room temperature. The protein was precipitated once more with methanol and finally dissolved in 50 μL of 50 mM ammonium bicarbonate, pH 7.0. The protein (400 μg) was incubated with trypsin (2 μg) overnight at 37°C. Samples of the digest (50 μL) were processed for LC-MS/MS analysis as described below.

Time-Dependent Modification of HSA by CBZE in Vitro. CBZE (50 mM) freshly dissolved in methanol and diluted in phosphate buffer (1:4, v/v) was incubated with HSA (1 mM, 40 μL) in phosphate buffer at 37^{o}C (final CBZE concentration, 5 mM). The final concentration of methanol was 10% (v/v). Aliquots of 80 μL were removed after 0.2, 0.5, 1, 3 and 6 h and processed for LC-MS/MS analysis of tryptic digests as described above.

Modification of HSA by Microsomally Generated Reactive Metabolites of CBZ. CBZ (50 mM) was freshly dissolved in methanol and then diluted 1:5 with phosphate buffer. The CBZ (final concentration, 1 mM) was co-incubated with either human liver microsomes (2 mg protein/mL) or Supersomes (20 nM P450), NADPH (1 mM), $MgCl_2$ (5 mM) and HSA (0.6 mM), in phosphate buffer for 2 h at 37^{o}C (final methanol concentration, 2% v/v). The incubations were then ultra-centrifuged at 105,000 *g* for 30 min at 4^{o}C. HSA was recovered from the supernatant by immunoaffinity column chromatography using a PerSeptive Biosystems Vision Workstation (Applied Biosystems, Foster City, CA) as described previously.[41] The HSA was eluted with 12 mM HCl, the eluate neutralized promptly using 1 M Tris buffer (pH9), and protein precipitated with nine volumes of ice-cold methanol. The protein was re-suspended in 50 µL of phosphate buffer and processed for LC-MS/MS as described above. Elution of the HSA conjugates under mild conditions - at room temperature, using a low mM acid concentration and relatively brief exposure to the eluent – prevented materially significant degradation of the conjugates.

Isolation of HSA from Patients' Plasma Samples by Resin Binding. Plasma samples (100 µL) were thawed at room temperature on the morning of analysis, and HSA was isolated from a 50 µL aliquot using a Pierce albumin depletion kit (Thermo Fisher Scientific, Paisley, United Kingdom) according to the manufacturer's instructions. The isolated HSA was reduced with 10 mM dithiothreitol (15 min) before alkylation with 50 mM iodoacetamide (15 min) at room temperature. An Amicon Ultra-0.5 mL centrifugal filter unit with Ultracel-50 membrane spin columns (Millipore, Watford, United Kingdom) was used to concentrate the albumin. The albumin samples were added to the spin column and centrifuged at 9200 *g* for 20 min. The columns were then washed with 400 µL of LCMS-grade water at 9200 *g* for 20 min. LCMS-

grade water (50 μL) was used to elute the albumin from the spin column. The protein content was determined using the Bradford assay. All the albumin solutions were normalised to 100 pmol/μL and 2 μg of trypsin was added to each sample before overnight incubation at 37ºC (16 h). After trypsin digestion, incubations were diluted to 5 pmol/μL in 2% acetonitrile/0.1% formic acid and synthetic K541 tryptic peptide of HSA (ATKEQLK) was added as internal loading standard such that its final concentration was 2.5 pmol/μL. Aliquots were analysed by LC-MS/MS as described below.

Mass Spectrometric Characterisation of Covalently Modified Proteins. In order to characterise all potential [O]CBZ- and CBZ-modified peptides, samples of HSA and GSTP tryptic digests were analysed firstly using a Triple TOF 5600 instrument (Sciex, Framingham, MA) by previously established methods.[38] Modified peptides were identified by filtering for specific fragment ions in PeakView 1.2.0.3 (Sciex) and manual inspection of the spectra.

Tryptic digests of GSTP and HSA (50 μL) were desalted using C18 Zip-Tips (Millipore, Watford, United Kingdom) according to the manufacturer's specifications. Eluates of the samples were dried in a rotational vacuum concentrator, and then reconstituted in 2% acetonitrile/0.1% formic acid (v/v). For the semi-quantitative analysis of [O]CBZ-modified peptides, aliquots (2.4−5.0 pmol) of the solutions were delivered into a QTRAP 5500 (Sciex) fitted with a NanoSpray II source by automated in-line liquid chromatography (UltiMate 3000 HPLC System, 5 mm C18 nanoprecolumn and 75 μm × 15 cm C18 PepMap column; Dionex, Sunnyvale, CA) via a 10-μm inner diameter PicoTip (New Objective, MA). A gradient from 2% acetonitrile/0.1% formic acid (v/v) to 50% acetonitrile/0.1% formic acid (v/v) in 60 min was applied at a flow rate of 300 nL/min. The ion spray potential was set to 2,200−3,500 V, the nebulizer gas to 19, and the interface heater to 150 °C. Spectra were acquired automatically in positive-ion mode using information-dependent acquisition powered by Analyst 1.5 software, across mass ranges of 400−1000 amu in MS and 100−1000 amu in MS/MS. The five most intense ions in each acquisition were selected for MS/MS, using a threshold of 5000 counts per second, with dynamic exclusion for 30 s and rolling collision energy.

For the MRM detection of drug-modified peptides, transitions specific for [O]CBZ- and CBZ-modified tryptic peptides of GSTP and HSA were selected as follows: mass/charge ratio values were calculated for all possible peptides containing a histidine, lysine or cysteine residue; to those values were added the mass of the proposed hapten (for CBZE and an arene oxide that has not rearomatized after its reaction with the protein, 252 amu; for an arene oxide that has rearomatized after its reaction, 234 amu); and finally the precursor ion masses were paired with the mass of a fragment ion that was expected to be a generic fragment of the predicted adducts, namely the hydroxydibenzoazepinyl species of m/z 210. MRM transitions were acquired at unit resolution in both the Q1 and Q3 quadrupoles to maximize specificity. They were optimized for collision energy and collision cell exit potential, and the dwell time was 50 ms. MRM survey scans were used to trigger enhanced product ion scans of [O]CBZ-modified peptides, with Q1 set to unit resolution, dynamic fill selected, and dynamic exclusion for 20 s. Total ion counts were determined from a second aliquot of each sample analysed by conventional LC-MS on the same instrument, and were used to normalize sample loading on the column. Relative quantification of modified tryptic peptides of protein recovered from in vitro incubations was performed by comparing the intensities of the MRM peaks for each of the modified residues normalized against total ion counts across samples. In the case of HSA isolated from patients, the relative quantification of modified peptides was achieved by comparing the intensities of the MRM peaks for each of the modified residues. The K541 peptide, which was detected as the transition m/z 817 / m/z 517, was employed to normalise the L-MS/MS loading of tryptic peptides across samples as described previously.[43] Data were analysed using Analyst software (Sciex).

Quantification of CBZ and its Metabolites in Human Plasma. The validated LC-MS/MS method of Breton et al. for measuring CBZ, CBZE, CBZ-DHD, 2-OH-CBZ and 3-OH-CBZ in human plasma,[23] which used 2-Me-CBZ as internal standard, was adapted to assay single plasma samples from fifteen epilepsy patients on established CBZ therapy.

Aliquots of stored plasma samples (300 μL), and the calibration and quality control standards (300 μL), were deproteinized with acetone (300 μL). The supernatants were evaporated to dry residue in a

rotational vacuum concentrator, and reconstituted in acetone and water (1:1, v/v; 300 μL). For the measurement of CBZ, CBZE and CBZ-DHD, reconstituted supernatant (20 μL) was injected onto a Zorbax C-8 pre-column and Eclipse XDB-C8 column (150 × 4.6 mm, 5μm) connected to a 1260 Infinity LC system (Agilent Technologies, Waldbronn, Germany) and 4000 Qtrap. Analytes were eluted at room temperature with an accelerated gradient program of acetonitrile (15% to 27% over 6 min; 27% to 32% over 5 min; 32% to 60% over 0.1 min; 60% for 2.9 min; 60% to 15% over 0.1 min; 15% for 2.4 min) in ammonium formate (2 mM, pH 3.8) at 1.0 mL/min. Instrument management and data processing were accomplished through Analyst 1.5.2 software (Sciex).

For the measurement of 2OH-CBZ and 3OH-CBZ the reconstituted supernatant (100 μL) was centrifugally filtered at 450 *g* for 15 min at 4ºC through a 0.45-μm hydrophilic PTFE filter plate (Merck Millipore, Watford, United Kingdom) to remove any remaining protein. The filtered supernatant (5 μL) was injected onto a 2.5 μM Xbridge BEH C18 VanGuard pre-column and Waters 2.5 μM Xbridge BEH C18 column (2.1 mm x 150 mm; Waters Limited, Herts, United Kingdom) connected to an UltiMate 3000 HPLC system and 4000 QTrap. Analytes were eluted at 40 ^{o}C with a gradient program of acetonitrile (15% to 20% over 2 min; 20% to 25% over 2 min; 25% to 30% over 2 min; 30% to 60% over 2 min; 60% for 4 min; 60% to 15% over 0.1 min; and 15% for 6.9 min) in ammonium formate (2 mM, pH 3.8) at 0.2 mL/min. Instrument management was achieved using the Chromeleon 6.80 software (Dionex Corporation), linked using Dionex chromatography MS link version 2.7.0.2551 to Analyst 1.5.1 software. Data processing was accomplished through Analyst 1.5.1 software. The mass spectrometer settings are listed in Tables S3 and S4. MRM chromatograms for the analytical standards and 2-Me-CBZ are shown in Figures S2 and S3 of the Supporting Information. Representative multi-channel MRM chromatograms of the analytes in patients' plasma are shown in Figures S4 and S5. The assay characteristics are summarized in Table S5. The absence of mass spectrometric (M+H parent ion) interference from the co-administered drugs (Table S6) and their known human plasma metabolites was established by literature searches for metabolite structures.

RESULTS

Identification of the Microsomally Generated Reactive Metabolites of CBZ by Trapping as GSH Conjugates. As reported previously, two isomeric *S*-glutathione conjugates of CBZE (CBZE-SG1 and CBZE-SG2) were produced by incubation of CBZE with GSH[25] and by incubation of CBZ, NADPH and GSH with human liver microsomes.[29] These two conjugates were also produced in the incubations of CBZ with all three of the CYP enzymes. The microsomal incubations yielded an additional *S*-glutathione conjugate that was more polar, and consistently less abundant, than the isomeric metabolites derived from CBZE (Figure 1). By a process of elimination, the polar product was deduced to be a conjugate of an arene oxide that had not undergone the spontaneous dehydration and rearomatization customarily but not invariably associated with such thiol-addition products.[45, 46] Bu et al. came to the same conclusion.[29] A rearomatized GSH conjugate, at m/z 542, was not detected. The proportions of the three GSH conjugates generated by CYP3A4 and CYP2E1 were similar but a relatively higher proportion of the more polar singleton conjugate was produced by CYP2B6 (Table 1). All three P450 enzymes generated CBZE, 2OH-CBZ and 3OH-CBZ (data not shown); whereas previously CYP2B6 and CYP2E1 were only known to catalyse the aromatic oxygenation of CBZ.[47]

Characterisation of GSTP Modified by CBZE and Microsomally Generated Metabolites of CBZ. A CBZE adduct (Δ 252 amu) was identified at Cys47 of GSTP after the protein was incubated with synthetic 10,11-epoxide (Figure 2). No adducts were detected at Cys14, Cys101 and Cys169. An [O]CBZ metabolite adduct was identified at Cys47 of GSTP that was isobarically consistent with the adduct formed when GSTP was reacted with synthetic CBZE. LC-MS/MS analysis of the tryptic digests of GSTP recovered from incubations of CBZ with human liver microsomes and Supersomes found the unmodified Cys47-containing ^{45}ASCLYGQLPK^{54} peptide (+2 ion at *m/z* 540.3). Modification of Cys47 by one or more [O]CBZ species (CBZE and/or one or more arene oxides; the adduct's product ion spectrum does not enable a more precise chemical assignment) resulted in a doubly charged peptide ion at *m/z* 666.3 that eluted at 37 min, corresponding to a mass increase of 252 amu compared with the mass of the unmodified peptide. The sequence of the proposed [O]CBZ-modified peptide, ^{45}ASC*LYGQLPK^{54}, was confirmed by the peptide's

product ion spectrum (Figure 2). An abundant ion at *m/z* 210.22 corresponded to isomeric fragments derived from synthetic CBZE, 2OH-CBZ and 3OH-CBZ, (Table S2), providing the first-line evidence of the adduct's structure (Figure 2). The b3* ion (*m/z* 514.2) and the immonium ion derived from cysteine (*m/z* 329.2), with an adduction of 252 amu, confirmed the addition of a [O]CBZ species to Cys47. Metabolism of CBZ to CBZE, 2OH-CBZ and 3OH-CBZ was confirmed in all of the human liver microsome incubations (data not shown); indicating the GSTP was probably exposed to arene oxides as well as to CBZE.[48]

Characterisation of Modification of HSA by CBZE In Vitro. Following the incubation of HSA with synthetic CBZE a triply charged ion of *m*/z 717.7 was detected by LC-MS/MS, corresponding to an adduct derived from the tryptic peptide ^{145}RHPYFYAPELLFFAK^{159} which was eluted at 70 min, with an additional mass of 252 amu. Abundant ions at *m/z* 180, 210, 236 and 253 correspond to fragments of the CBZE-derived moiety of the adduct, the proposed structures of which are shown in Figure 3A. The b4* (+2 ion at *m/z* 403.7), b5* (+2 ion at *m/z* 477.2), b6* (+2 ion at *m/z* 558.7) and b7* (+2 ion at *m/z* 594.3) ions all have the mass addition of 252 amu, indicating [O]CBZ modification at His146 (Figure 3C). The spectrum for the unmodified peptide ^{145}RHPYFYAPELLFFAK^{159} is available in the supplementary information as Figure S6. The His146 adduct was detected at all concentrations of CBZE and its relative abundance was positively correlated with increasing CBZE concentration (Figure 3D). The reaction between CBZE and HSA was first detected at 30 min and showed a positive trend towards greater relative abundance of adduct over time up to 6 h (Figure 3D). A second triply charged ion, of *m/z* 573.9, was detected by LC-MS/MS at 60 min, corresponding to an adduct derived from the tryptic peptide ^{337}RHPDYSVVLLLR^{348} with an additional mass of 252 amu (Figure S7). The b3* (+2 ion at *m/z* 378.7), b6* (+2 ion at *m/z* 504.7) and b7* (+2 ion at *m/z* 554.2) ions all have the mass addition of 252 amu confirming addition of CBZE to His338. However, the His338 adduct was detected only at the highest molar ratio (5:1), and only after at least 3 h incubation. No CBZE adduct was detected at Cys34 of HSA.

Characterisation of HSA Modified by Microsomally Generated Metabolites of CBZ. LC-MS/MS analysis of tryptic digests of HSA recovered from incubations with CBZ, NADPH and human liver

microsomes revealed [O]CBZ adducts at His146 (Figure 3E) and His338 that were indistinguishable from the adducts formed by the reaction of synthetic CBZE with HSA . There was also evidence for formation of CBZ adducts at His146 and His338, that is, a stable adduct produced by dehydration of a primary, dihydrohydroxyaryl, structure derived from an arene oxide. Figure 4A outlines the proposed mechanism for formation of an arene oxide adduct. A triply charged ion of *m/z* 711.7 detected at 63 min corresponded to an adduct of ^{145}RHPYFYAPELLFFAK^{159} with an additional mass of 234 amu (Figure 4B), and the highlighted b and y fragments confirm the peptide sequence. A second triply charged ion detected at 49 min corresponded to an adduct of ^{337}RHPDYSVVLLLR^{348} with an additional mass of 234 amu (Figure S8).

Detection of CBZ-Derived Covalent Modifications of HSA in Epilepsy Patients. The clinical details of the fifteen patients who donated plasma samples are outlined in Table S1. Five subjects donated two plasma samples on separate visits. In total 20 plasma samples were analysed. Eight of the subjects were male and subjects were aged between 27 and 75, with the total daily dose of CBZ ranging from 200 mg to 1600 mg. The timings of the blood samples relative to the last dose of CBZ ranged from 70 to 580 min. The same [O]CBZ-modified His146 peptide, ^{145}RH*PYFYAPELLFFAK^{159}, was identified in all twenty plasma samples (Figure 5A), and its relative amounts are illustrated in Figure 5B. There was 16-fold variability between the lowest level of [O]CBZ-HSA adduct (subject C009) and the highest (subject C036). The relative plasma levels of [O]CBZ-HSA adduct, and the concentrations of CBZ, CBZE, 2OH-CBZ and 3OH-CBZ, for each subject are available in Table S7. Correlations between the relative plasma levels of the [O]CBZ-modified HSA adduct and either drug dosage or the plasma concentrations of CBZ and its metabolites were undertaken. There was a moderate positive correlation between total daily CBZ dosage and the relative amount of the adduct (r^2 = 0.39, p = 0.003). The strongest positive correlation was observed between CBZ plasma concentrations and the relative amount of adduct (r^2 = 0.44, p = 0.002). Moderate positive correlations were also obtained between the relative amount of adduct and plasma CBZE concentrations (r^2 = 0.35, p = 0.006) and between adduct levels and total plasma concentrations of the monoxygenated ([O]CBZ) metabolites (r^2 = 0.36, p = 0.005). The weakest positive correlation was observed

between the combined plasma concentrations of 2OH- and 3OH-CBZ and the relative amount of adduct (r^2 = 0.26, p = 0.021) (Figure 6).

Discussion

This study demonstrates that microsomally generated electrophilic monoxygenated metabolites of CBZ are able to form covalent adducts with co-incubated soluble proteins - human GSTP and HSA - as well as with microsomal proteins[15, 26, 27] and GSH (Scheme 2).[25, 29] Reactive metabolite trapping by exogenous protein incubated with hepatic microsomes has been employed previously to identify the metabolites and characterize the protein adducts. Yukinaga et al. captured reactive intermediates of acetaminophen and raloxifene on Cys47 of human GSTP.[49] Damsten et al. produced an adduct of *N*-acetyl-p-benzoquinone imine (NAPQI), the reactive metabolite of acetaminophen, on Cys34 of HSA by incubating whole plasma with the drug and rat liver microsomes.[50]

Notwithstanding the chemical similarities of the synthetic CBZE-Cys47 and biogenic [O]CBZ-Cys47 adducts, precise structural assignment of the latter was not achievable. When the reactive metabolites of CBZ produced by human liver microsomes were sampled with GSH, which enabled chromatographic resolution and a more confident identification of conjugate structures, confounding evidence of arene oxides as well as CBZE was obtained. The distinctive polar dihydrohydroxyCBZ-*S*-glutathione conjugate ([O]CBZ-SG) resolved from the two CBZE derivatives was consistently less abundant but effectively precluded exclusive assignment of the [O]CBZ residue in the [O]CBZ-Cys47 adduct. However, LC-MS/MS analysis of the microsomal incubations failed to deliver supporting evidence of an arene oxide, in the form of a CBZ-*S*-glutathione conjugate generated by indicative rearomatization of an intermediate arene oxide derivative.[45] An alternative, enzymic, approach to identifying a Cys47 adduct of an arene oxide was attempted. This was based on a presumed selectivity of benzylic and aromatic oxygenation of CBZ by P450 enzymes. CYP3A4 is the predominant catalyst of 10,11-epoxidation in human liver microsomes, and otherwise only CYP2C8 was known to have this activity,[51] whereas CYP2B6 is the most active aromatic hydroxylase.[47] Incubations with Supersomes confirmed benzylic and aromatic oxygenation by CYP3A4 but

also revealed 10,11-epoxidation by CYP2E1 and CYP2B6. Nevertheless, comparison of the aromatic hydroxylase activity of the three P450 enzymes[47] demonstrated positive association with the relative abundance of [O]CBZ-SG. It has been proposed that a reactive arene oxide intermediate is formed during hydroxylation of CBZ.[15, 29] The greater formation of [O]CBZ-SG by CYP2B6, the isoform with greatest aromatic hydroxylase activity, is therefore consistent with the attribution of this conjugate to an arene oxide derivative. What appeared to be multiple [O]CBZ-SG and CBZ-SG adducts were detected in isolated rat hepatocytes.[52] Several examples of stable dihydrohydroxyaryl-*S*-cysteinyl adducts of GSH,[46, 53] other peptides[46, 54] and proteins[55] are known, indicating a structure of this type is less likely to rearomatize than might have been thought. Consequently it is a reasonable proposition that some of the [O]CBZ-*S*-Cys47 adducts generated by human liver microsomes and Supersomes could have been derived from an arene oxide.

Two adducts, at His146 and His338, were identified on incubation of CBZE and HSA. The His146 adduct was detectable at a low molar ratio (0.1:1, CBZE:HSA) after 30 min. Analogous (isobaric) [O]CBZ adducts at His146 and His338 were formed in microsomal incubations that also generated CBZE, 2OH-CBZ and 3OH-CBZ in the presence of HSA. Two additional adducts at His146 and His338, having the form of CBZ-HSA, were found in the microsomal incubations of CBZ with HSA, and were assigned preliminarily to rearomatized derivatives of arene oxide adducts. If confirmed, this would represent the first chemical evidence of a CBZ arene oxide that has the ability to escape the microsomal microenvironment and modify soluble proteins. The His146 residue of HSA is located accessibly at the entrance to fatty acid binding site 1 in subdomain IB.[56] It reacts with diverse electrophilic species, including nevirapine metabolites,[36] abacavir aldehyde (which also reacts with Cys34),[37] and Δ^{12}-prostaglandin J2, an α,β-unsaturated ketone which functions as a regulator of inflammation.[56] The (-)-enantiomer of the benzylic *anti*-diol epoxide of benzo[a]pyrene, which has certain structural similarities to CBZE, combines with HSA exclusively at N^{τ} of His146, retaining the dihydrohydroxyaryl structure.[57] Although the Cys34 residue is conventionally regarded as the predominant site of HSA's reactions with organic electrophiles,[58] and is claimed to be more reactive than His146,[59] no cysteine adducts were detected with either synthetic CBZE or [O]CBZ

metabolites. However, a number of other thiophilic compounds have also failed to react with Cys34 on HSA.[56, 60] Commercial preparations of HSA often contain very high levels of mixed disulfides at Cys34,[61] potentially reducing its ability to react with electrophilic metabolites and leading to a failure to detect adducts. However, we have previously used the commercial HSA preparation selected for the present studies and readily detected adducts of 12-sulfoxynevirapine with Cys34 as well as with three histidine residues (including His146).[36] Incubation of CBZE (final concentration, 0.8 mM) with fresh human plasma (37 ^{O}C, 3 h), in which the fraction of sulfhydryl HSA is approximately 65%, generated adducts at His146 and His338 but not at Cys34 (data not shown). It is therefore reasonable to attribute the absence of a detectable [O]CBZ adduct at Cys34 firstly to an inability of the epoxide to react with Cys34 rather than the absence of a nucleophilic (thiol/thiolate) side chain. While molecular dynamic simulations and docking studies have shown that the nucleophilic Cys34 thiolate is more accessible than the residue's thiol form,[62] the cavity lined by Cys34 is evidently too hindered to allow alkylative access by CBZE. Thus the side chain-selective adduction of GSTP and HSA by CBZE is characterized by a distinct duality: only occurring at cysteine (one of four; Figure 2B) in GSTP notwithstanding the protein has two histidines; and only at histidine (two of sixteen) in HSA notwithstanding the protein has a free cysteine of considerable nucleophilicity (Figure 3B). The selectivity of HSA's haptenation is nonetheless highly compound-dependent; vinyl sulfones[66] and abacavir aldehyde[36] react at both Cys34 and His146.

If the skin reactions associated with CBZ[63, 64] are initiated by local protein haptenation, the haptenating species might be CBZE formed in the liver and delivered via the blood.[23] However, protein-reactive metabolites of CBZ might also be produced locally. Cutaneous hypersensitivity to sulfonamides is similarly thought to be initiated by reactive drug metabolites.[65] Intracellular protein adducts were detected when sulfamethoxazole or dapsone was incubated with human epidermal keratinocyte.[66-68] Thus keratinocytes are able to bioactivate drugs and form drug-protein adducts which may represent the antigenic determinants for T cells. Exceptionally, bioactivation in keratinocytes was mediated by flavin-containing monoxygenases and peroxidases with limited involvement of P450.[67, 68] Nevertheless human skin expresses multiple P450 enzymes, including CYP3A4 and CYP2B6.[69] Levels of CYP2B6 mRNA demonstrated the largest inter-

individual variation, with CYP2B6 mRNA undetectable in some skin samples. Consequently metabolism of CBZ to reactive [O]CBZ species could occur in keratinocytes and there would be variation between patients in the production of [O]CBZ. Patients hypersensitive to CBZ may generate higher levels of [O]CBZ, overwhelming detoxification pathways and leading to keratinocyte death and hypersensitivity.

For the first time [O]CBZ-modified protein was also detected in vivo, in patients prescribed standard CBZ therapy for epilepsy and without any indications of drug toxicity; including any indications of the neurotoxicity that has been associated with unusually high plasma concentrations of CBZE.[70] The His146 [O]CBZ-HSA adduct was identified in plasma samples from all of the 15 patients. There was both inter- and intra-patient variation in relative abundance of the adduct. Intra-patient variation in relative adduct abundance implies that the levels of [O]CBZ-HSA are likely to be dependent on parameters other than the size and timing of the dose. It is possible that all patients who receive CBZ therapy have [O]CBZ-HSA adducts in their circulation, irrespective of the imminence or presence of hypersensitivity, implying that other factors need also to be present to trigger a hypersensitivity reaction. Similar analyses conducted in patients receiving flucloxacillin therapy also identified parent drug- and metabolite-modified HSA in all (n=8) tolerant patients.[40] These observations suggest that circulating drug-modified HSA alone might be generically insufficient to trigger hypersensitivity. Indeed, it is thought that a complex history of genetic and non-genetic patient factors determine the frequency and severity of drug hypersensitivity.

CBZE-HSA adducts appeared detectably in vitro from 30 min and increased over time. The half-life of human albumin is 19 days, so it is expected that as patients commence CBZ therapy and titrate their dosages the levels of CBZE-HSA adduct will also increase. It is possible that hypersensitivity reactions to CBZ develop only when a critical threshold of adducts is exceeded. The time taken to reach that threshold could partially explain the two to six week delay between starting CBZ therapy and manifestation of hypersensitivity (Figure 3D).[71] Cystic fibrosis patients receive multiple intravenous courses of antibiotics because of recurrent infections and are known to have a high incidence of piperacillin hypersensitivity (30-50%).[72] Studies in those patients demonstrated that as the plasma concentration of piperacillin is increased

the number of modified residues on albumin increases, generating a greater number of epitopes that could be recognised by the immune system.[73] Similarly, the current study has identified a moderate but significant positive correlation (r^2 = 0.39, p= 0.003) between relative abundance of [O]CBZ-HSA adduct and the dosage of CBZ. The strongest positive correlation was observed between relative abundance of [O]CBZ-HSA and plasma CBZ concentrations. However, comparisons of the relative amount of [O]CBZ-HSA with plasma CBZE concentrations and the total concentrations of plasma [O]CBZ metabolites (CBZE, 2OH-CBZ and 3OH-CBZ) unexpectedly resulted in weaker positive correlations. In particular, the persistent circulation of CBZE in plasma suggested a greater positive correlation with epoxide concentration was to be expected. This finding implies the [O]CBZ-HSA adducts were not derived substantially from the intravascular reaction of CBZE with HSA. Admittedly the assay of plasma [O]CBZ-HSA adduct was only semi-quantitative, because a CBZE-modified His146 tryptic peptide was not available for calibrated measurement of the adduct. [^{14}C]CBZE is known to form covalent adducts with protein in human plasma ex vivo[25] but apart from HSA, identified here, none of the modified proteins has been characterized. Therefore the present estimate of HSA's adduction should be regarded as the minimum amount of [O]CBZ adducts formed.

Synthetic CBZE and microsomal incubations of CBZ with HSA generated a second [O]CBZ-HSA adduct, at His338; which together with His146 and His242 is also adducted by the electrophilic sulfate of nevirapine.[36] However, no adduct other than the [O]CBZ-HSA at His146 was observed in patient samples because plasma levels of CBZE are unlikely to reach the molar ratios tested in vitro. The greatest concentration of CBZE observed in patients was 5.2 µg/mL (0.02 mM) and when compared with a typical plasma albumin concentration of 0.6 mM results in a molar ratio of 0.03:1 (CBZE:HSA) in comparison with molar ratios of 0.1:1 to 5:1 (CBZE:HSA) tested in vitro. Although unlikely it is possible that a small fraction of patients form a second hapten during CBZ therapy. Russell et al. found a plasma CBZE concentration of 54 µg/mL (214 µM) after multiple medication overdose, including 17.8 g of CBZ, which would translate to molar ratios in the range of the in vitro incubations.[70] The relationship between plasma levels of drug/metabolite-protein adduct and susceptibility to hypersensitivity is poorly understood and warrants further investigation.

It has been suggested that CBZ hypersensitivity is mediated through a PI mechanism.[10] However, all of the evidence is derived from experiments with isolated cells which cannot replicate the complexity of in vivo situations; where the extracellular drug concentrations might deviate substantially from those in tissues and the T-cells are not exposed to metabolites produced by cell types expressing P450 abundantly. Additionally, genetic variation in the activity of drug metabolism enzymes might lead to critical inter-individual differences between the levels of immunologically active metabolites.[74] *HLA-B*15:02* and *HLA-A*31:01* have both been associated with specific phenotypes of CBZ hypersensitivity reaction but both associations have very low positive predictive values,[75] implying that factors other than HLA genotype are involved in pathogenesis of the hypersensitivity. The PI mechanism alone is unable to account for these findings. Future studies need to focus on assay systems that enable the generation of reactive metabolites and incorporate drug-modified peptides to determine their ability to elicit T-cell responses.[73]

In conclusion, this study has provided the first chemical evidence for the microsomal production of [O]CBZ species that are able to escape the microsomal domain to react covalently with exogenous soluble proteins. If those species are equally able to escape from the endoplasmic reticulum of living cells they might haptenate numerous intracellular proteins. Most importantly, this study has demonstrated the presence of circulating [O]CBZ-modified HSA in patients who were receiving standard CBZ therapy. These findings have provided new insights into potential molecular mechanisms associated with toxicity of CBZ. Similar to the multiple protein adducts formed by nevirapine,[36] the great variety of adducts formed by CBZ's metabolites may account for the diversity of the organ toxicity of CBZ. Further studies are warranted to determine the biological effects of CBZ-modified proteins and peptides in in vitro models using well-defined synthetic forms of the [O]CBZ and CBZ adducts discovered in this study. Definition of a quantitative relationship between CBZ antigens formed in tissues and the activation of tissue-specific T cells could pinpoint the causal metabolites involved in the rare but serious toxicity associated with CBZ in either skin or liver.

ASSOCIATED CONTENT

Supporting Information

Clinical, medication and blood sampling information for the patients; chromatographic and analytical parameters of the plasma drug and metabolite assays; mass spectrometer settings for the assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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M.P., B.K.P. and A.G.M supervised the project; V.L.M.Y., X.M, J.L.M., R.E.J. and A.G.M. designed the experiments; X.M. prepared the peptide reagent; V.L.M.Y., X.M., R.E.J. and P.M. performed the experiments; all of the authors contributed to the writing of this paper and gave approval to the final manuscript.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

CBZ, carbamazepine; HLA, human leukocyte antigen; PI, pharmacological interaction; TCR, T-cell receptors; GSH, glutathione; carbamazepine 10,11-epoxide, CBZE; CBZE-SG1 and CBZE-SG2, carbamazepine 10,11-epoxide-glutathione adducts 1 and 2; HSA, human serum albumin; GSTP, glutathione *S*-transferase pi; [O]CBZ, monoxygenated (carbamazepine) metabolites; LC-MS, liquid chromatography-mass spectrometry; 2-Me-CBZ, 2-methylcarbamazepine; CBZ-DHD, carbamazepine-10,11-trans-dihydrodiol; 2-OH-CBZ, 2-hydroxycarbamazepine; 3-OH-CBZ, 3-hydroxycarbamazepine; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MRM, multiple reaction monitoring.

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Table 1. Relative Proportions of the Three Isomeric Dihydrohydroxy-*S*-glutathione Conjugates Formed from CBZ by human liver microsomes and Supersomes

enzymic preparation	ratio of GSH adducts ([O]CBZ-SG: CBZE-SG1: CBZE-SG2)[a]	aromatic hydroxylase activity (pmol/nmol P450/min)[b]
Human liver microsomes	7.0 : 45.2 : 47.8	NA
CYP3A4	10.3 : 42.5 : 47.2	72.5
CYP2B6	25.5 : 34.5 : 40.0	186
CYP2E1	13.1 : 40.6 : 46.3	38

[a]Relative areas of peaks in *m/z* 560 → 431 MRM chromatograms acquired by LC-MS/MS (neutral loss of pyroglutamate from the protonated molecule). CBZE-SG1 and CBZE-SG2 are isomeric products of the reaction of CBZ 10,11-epoxide with GSH, identified by chromatographic and mass spectrometric comparisons with synthetic standards. [O]CBZ-SG is a GSH conjugate of unknown regiochemistry and stereochemistry assigned by process of elimination to a derivative of an arene oxide.

[b]Combined C-2 and C-3 CBZ hydroxylase activities of human P450 enzymes taken from Pearce et al.[47]

NA= not applicable.

Scheme Legends

Scheme 1. Proposed Major Pathways of Bioactivation of CBZ in Humans and the Enzymes Catalysing the Reactions[a]

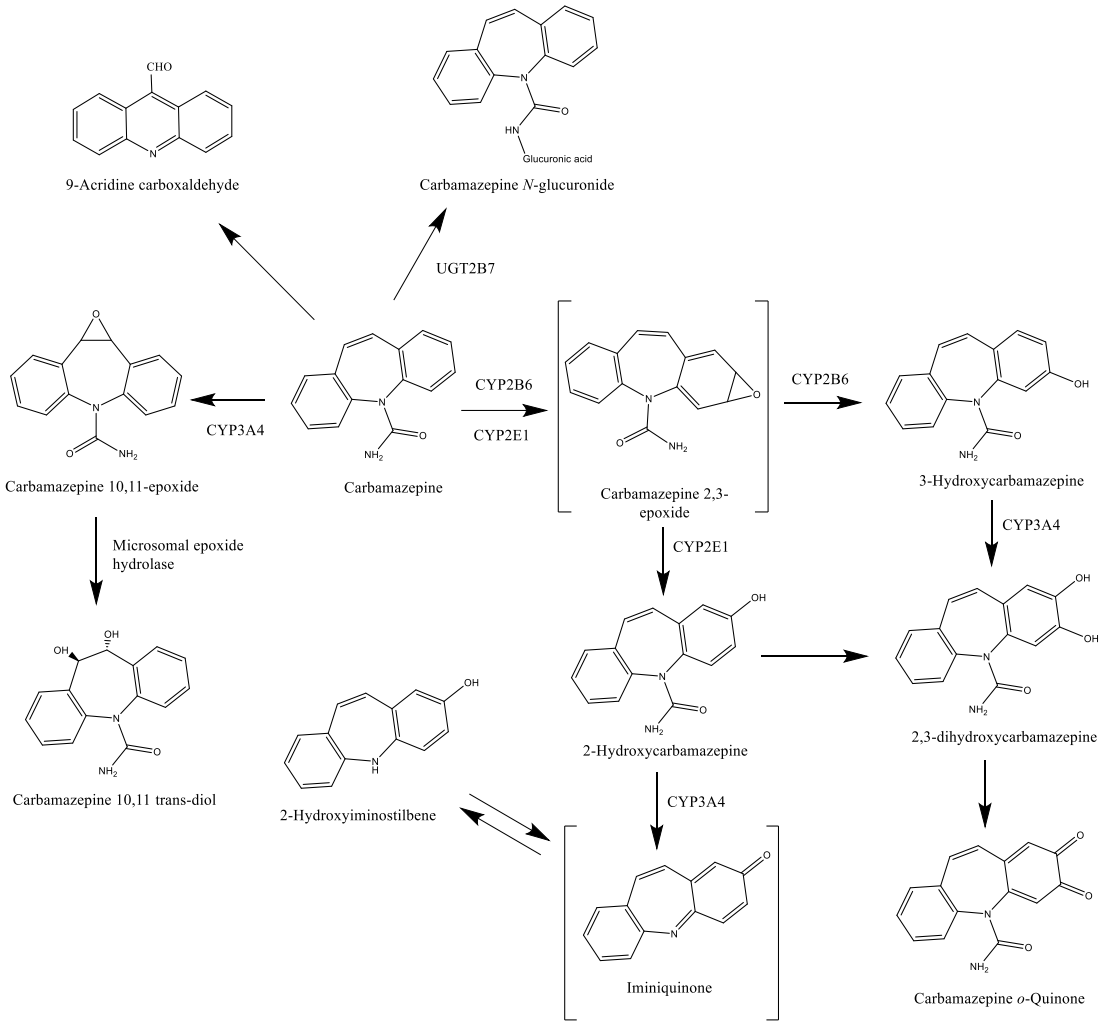
[a]The P450 enzymes shown are those reported to be the principal catalysts of the biotransformations in question. However, it was found that CY2B6 and CYP2E1, as well as CYP3A4, catalyze the 10,11-epoxidation of CBZ in vitro. The depiction of the 2,3-arene oxide as the sole product of aromatic epoxidation is purely representational; the number of arene oxides formed is unknown. Only protein adducts of the major reactive intermediates, namely the 10,11-epoxide and arene oxides, were considered during the present work.

Scheme 2. Pathways for the bioactivation of CBZ and formation of [O]CBZ adducts with GSTP, GSH and HSA in vitro and in humans[a]

[a]In human plasma samples, only HSA modified by [O]CBZ species at His146 was detected.

CBZE, carbamazepine 10,11-epoxide; HSA, human serum albumin; GSH, glutathione; GSTP, glutathione *S*-transferase pi; [O]CBZ, monoxygenated (carbamazepine) metabolites.

Scheme 1


9-Acridine carboxaldehyde
Carbamazepine N-glucuronide
Glucuronic acid
UGT2B7
Carbamazepine 10,11-epoxide
CYP3A4
Carbamazepine
CYP2B6
CYP2E1
Carbamazepine 2,3-epoxide
CYP2B6
3-Hydroxycarbamazepine
CYP3A4
Microsomal epoxide hydrolase
CYP2E1
Carbamazepine 10,11 trans-diol
2-Hydroxyiminostilbene
2-Hydroxycarbamazepine
2,3-dihydroxycarbamazepine
CYP3A4
Iminiquinone
Carbamazepine o-Quinone

Scheme 2

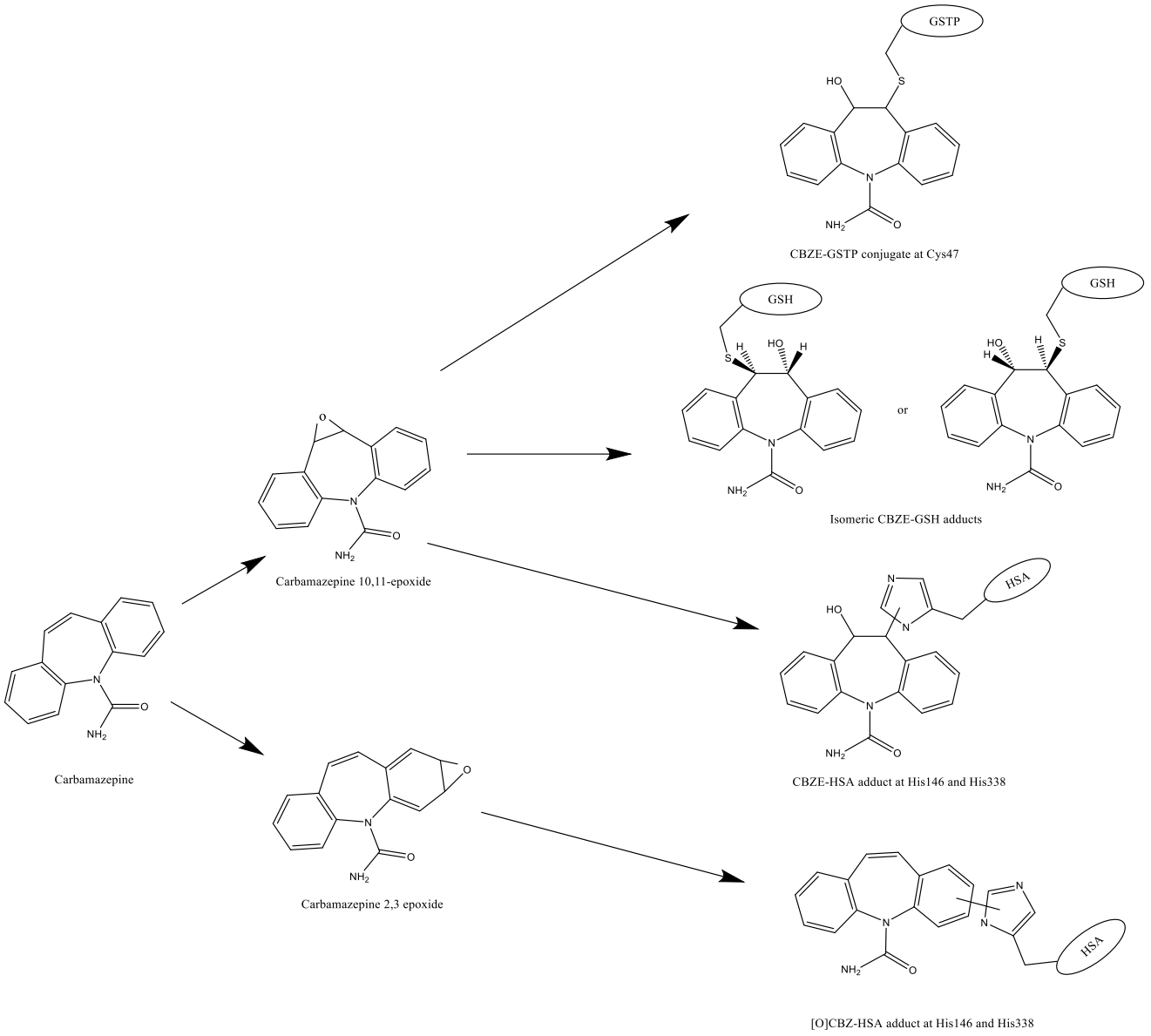

GSTP
HO
S
N
NH2
O
CBZE-GSTP conjugate at Cys47
GSH
H
HO
S
or
Isomeric CBZE-GSH adducts
Carbamazepine 10,11-epoxide
HSA
CBZE-HSA adduct at His146 and His338
Carbamazepine
Carbamazepine 2,3 epoxide
[O]CBZ-HSA adduct at His146 and His338

Figure Legends

Figure 1. Isomeric GSH adducts of reactive monoxygenated metabolites of CBZ that were formed from CBZ incubated with HLM and Supersomes. The thioether metabolites produced by CYP2B6 Supersomes are shown. CYP2B6 is the most active CBZ C-2 hydroxylase and the most active CBZ hydroxylase overall.[45] CBZE-SG1 and CBZE-SG2 were assigned by comparison with standards prepared from synthetic CBZE. The assignment of the third product to an adduct of CBZ arene oxide was based on inference.

Figure 2. Characterization of CBZE-GSTP adducts formed in vitro. The proposed general chemical structure and characteristic hapten fragment ion of the adduct formed by CBZE and GSTP (A); Ribbon diagram of human GSTP showing the locations of the four cysteine residues (red and yellow residues) [PyMol software **(**Schrödinger, Munich, Germany)]. Only Cys47 (red residue) was modified detectably, by synthetic CBZE in vitro and by monoxygenated CBZ metabolites (CBZE and/or CBZ arene oxide) in hepatic microsomal incubations. Cys14 and Cys101 are documented sites of alkylation by other electrophilic species,[32,33] whereas Cys169 is outstandingly unreactive[33] (B); The top panel is a representative MS/MS spectrum of the unmodified GSTP peptide ^{45}ASCLYGQLPK^{54}. The bottom panel is a representative MS/MS spectrum of the GSTP peptide ^{45}ASCLYGQLPK^{54} adducted at Cys47 by CBZE in direct incubation. A characteristic fragment ion derived from partial cleavage of the hapten is circled (C).

Figure 3. Characterization of CBZE-HSA adducts formed in vitro. The proposed general chemical structure and characteristic hapten fragment ions of the adduct formed by CBZE and HSA (A); Ribbon diagram of HSA showing the locations of the two residues adducted by CBZE in vitro: His146 and His338 (blue residues) [PyMol software (Schrödinger, Munich, Germany)]. The single free cysteine residue, Cys34, is shown as a red residue. It was not modified detectably, either in vitro or in vivo (B); representative MS/MS spectrum of the peptide ^{145}RHPYFYAPELLFFAK^{159}

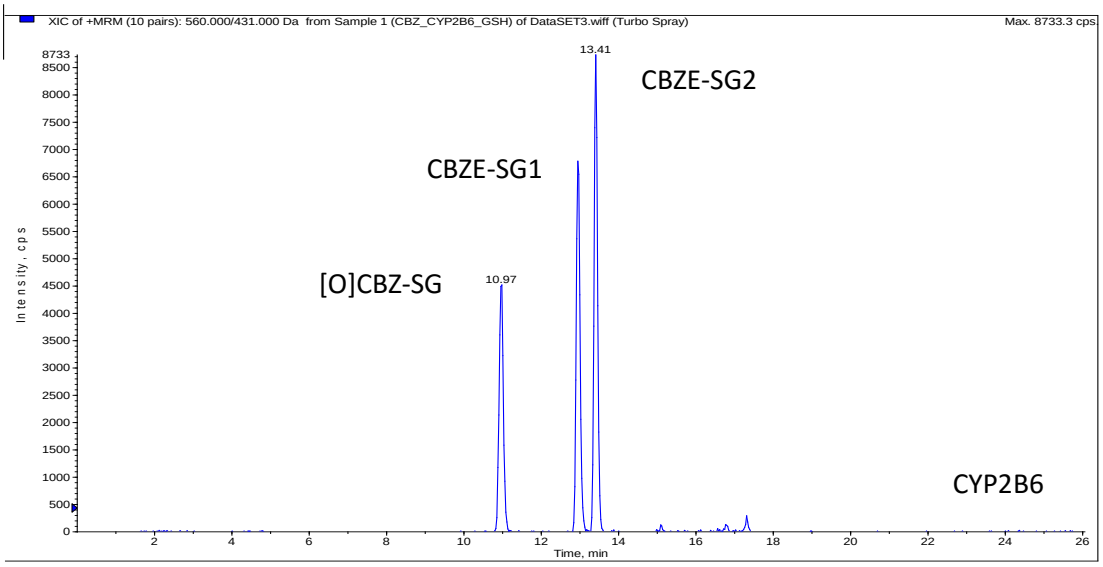
modified at His146 with CBZE; characteristic fragment ions derived from partial cleavage of the hapten are circled (C); the level of modification of the His146 peptide was concentration- and time-dependent (drug/protein molar ratio 5:1) (D); Representative MS/MS spectrum of the peptide ^{145}RHPYFYAPELLFFAK^{159} modified at His146 with [O]CBZ from the CYP3A4 Supersomes incubation; characteristic fragment ions derived from partial cleavage of the hapten are circled (E).

Figure 4. Characterization of the adducts of CBZ arene oxide and HSA formed by microsomal incubations. Proposed reaction of arene oxide metabolite with His146 and His338 of HSA; showing rearomatization of the primary dihydrohydroxy adduct (A); representative MS/MS spectrum of the peptide ^{145}RHPYFYAPELLFFAK^{159} modified at His146 by CBZ (considered to be the product of rearomatization of a dihydrohydroxy adduct of an arene oxide) (B)

Figure 5. LC-MS/MS analysis of modified HSA peptides identified in patients prescribed CBZ therapy. Representative MS/MS spectrum of the albumin peptide ^{145}RHPYFYAPELLFFAK^{159} modified at His146 by [O]CBZ (A); Relative quantitation of [O]CBZ-HSA adducts in patients' plasma samples. (b) denotes second plasma sample from the same patient at separate visit; data represents mean (n=3) and standard deviation (B).

Figure 6. Correlations between relative amounts of [O]CBZ-HSA adduct in patients' plasma samples and drug/metabolite concentrations. Total daily dosage (A); CBZ plasma concentration (B); CBZE plasma concentration (C); 2OH- and 3OH-CBZ plasma concentrations (D); total [O]CBZ plasma concentration (CBZE + 2OH-CBZ + 3OH-CBZ) (E).

Figure 1


XIC of +MRM (10 pairs): 560.000/431.000 Da from Sample 1 (CBZ_CYP2B6_GSH) of DataSET3.wiff (Turbo Spray)
Max. 8733.3 cps
13.41
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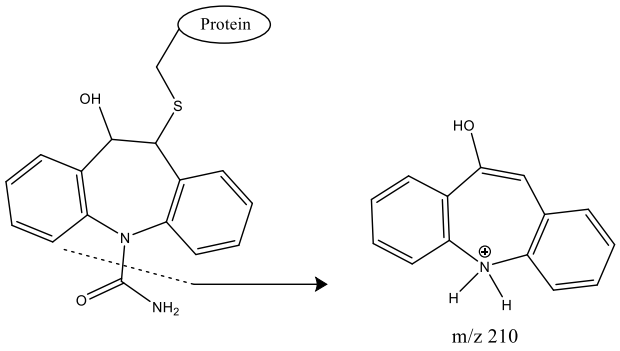
CBZE-SG1
[O]CBZ-SG
10.97
CYP2B6
Intensity, cps
Time, min

Figure 2

A


Protein
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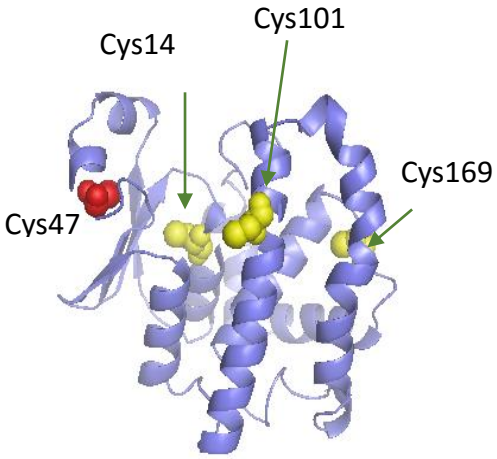
HO
N
H
H
m/z 210

B


Cys14
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Cys169
Cys47

C

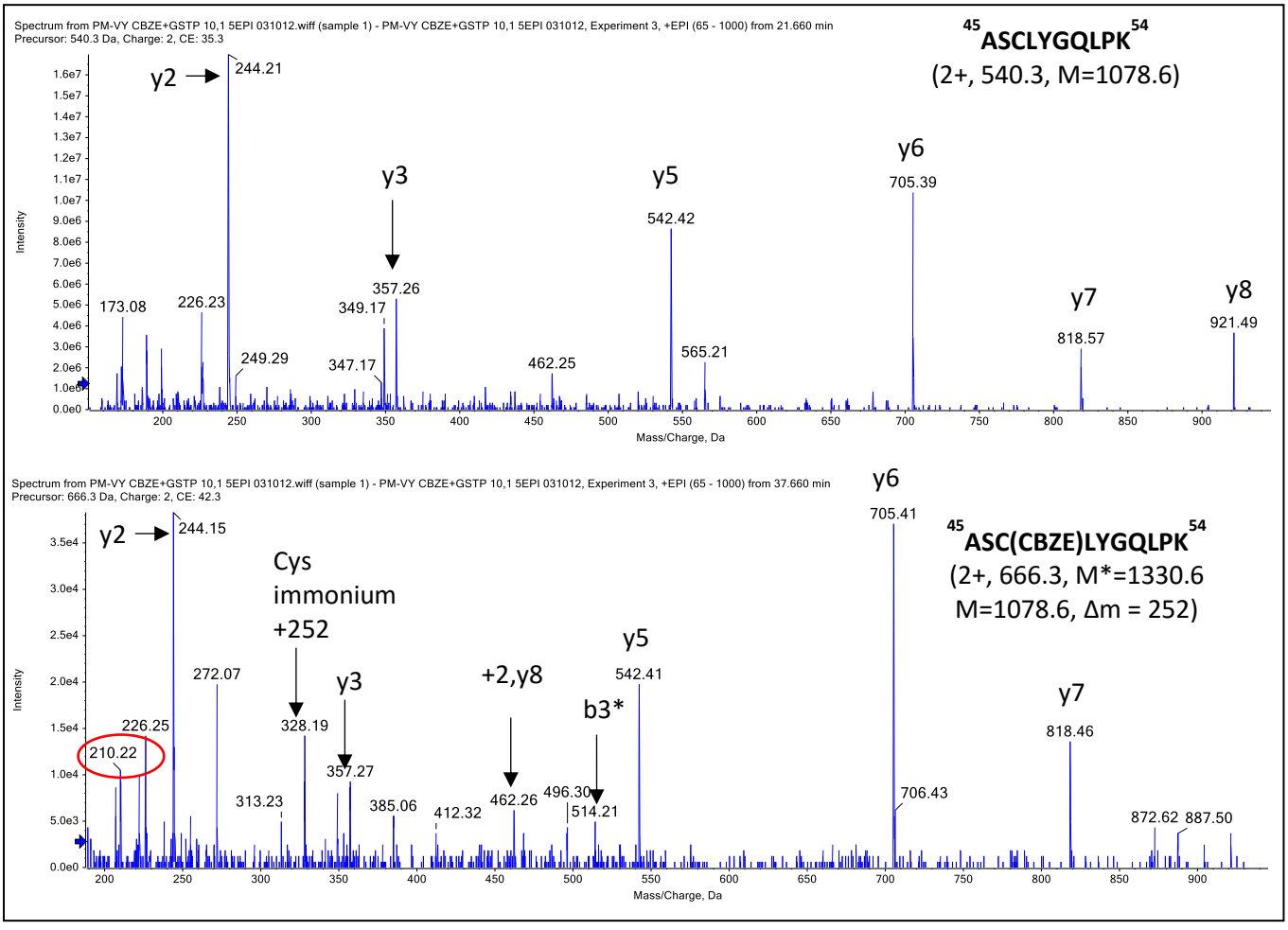
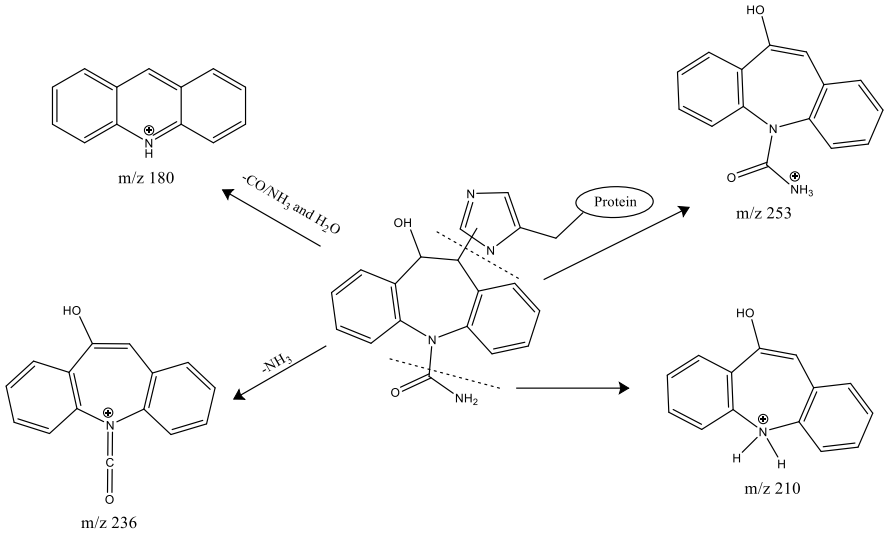
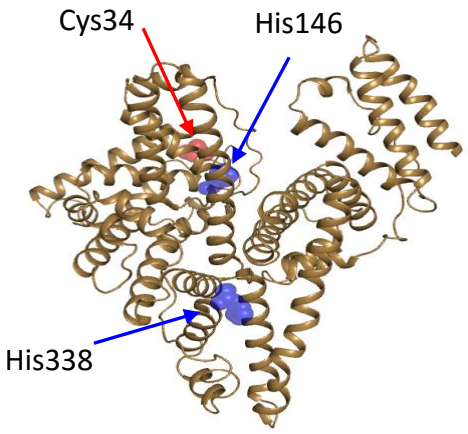

Spectrum from PM-VY CBZE+GSTP 10,1 5EPI 031012.wiff (sample 1) - PM-VY CBZE+GSTP 10,1 5EPI 031012, Experiment 3, +EPI (65 - 1000) from 21.660 min
Precursor: 540.3 Da, Charge: 2, CE: 35.3
45ASCLYGQLPK54
(2+, 540.3, M=1078.6)
y2
244.21
y3
357.26
y5
542.42
y6
705.39
y7
818.57
y8
921.49
173.08
226.23
249.29
347.17
349.17
462.25
565.21
Intensity
Mass/Charge, Da
Spectrum from PM-VY CBZE+GSTP 10,1 5EPI 031012.wiff (sample 1) - PM-VY CBZE+GSTP 10,1 5EPI 031012, Experiment 3, +EPI (65 - 1000) from 37.660 min
Precursor: 666.3 Da, Charge: 2, CE: 42.3
45ASC(CBZE)LYGQLPK54
(2+, 666.3, M*=1330.6
M=1078.6, Δm = 252)
y2
244.15
Cys
immonium
+252
y3
328.19
357.27
+2,y8
462.26
y5
542.41
b3*
496.30
514.21
y6
705.41
706.43
y7
818.46
872.62
887.50
272.07
226.25
210.22
313.23
385.06
412.32
Intensity
Mass/Charge, Da

Figure 3

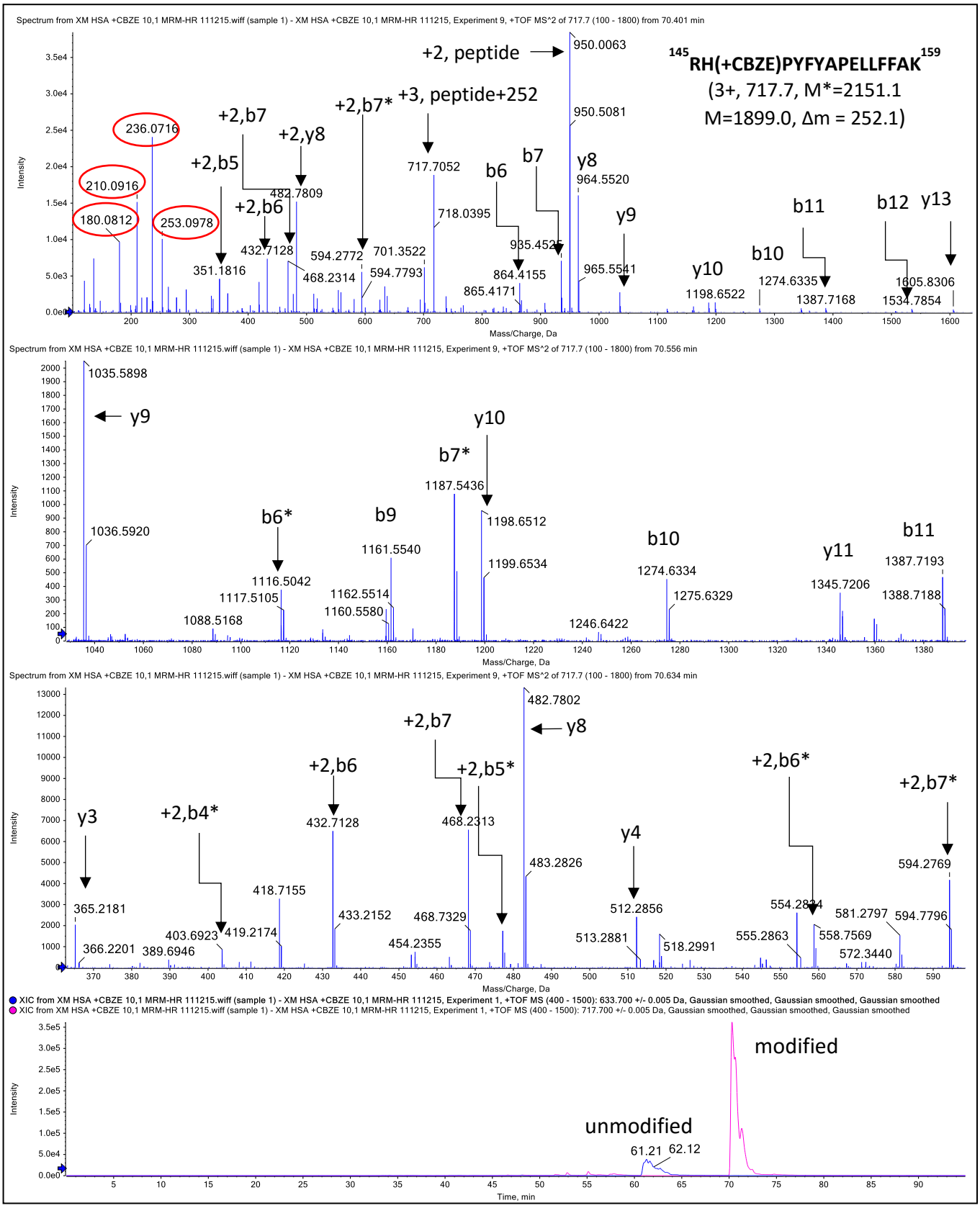
A


m/z 180
-CO/NH3 and H2O
Protein
m/z 253
-NH3
m/z 236
m/z 210

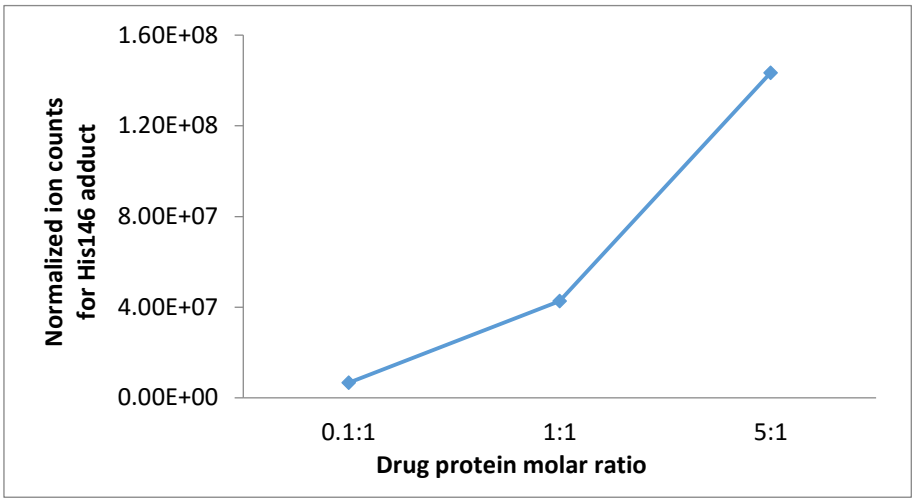
B

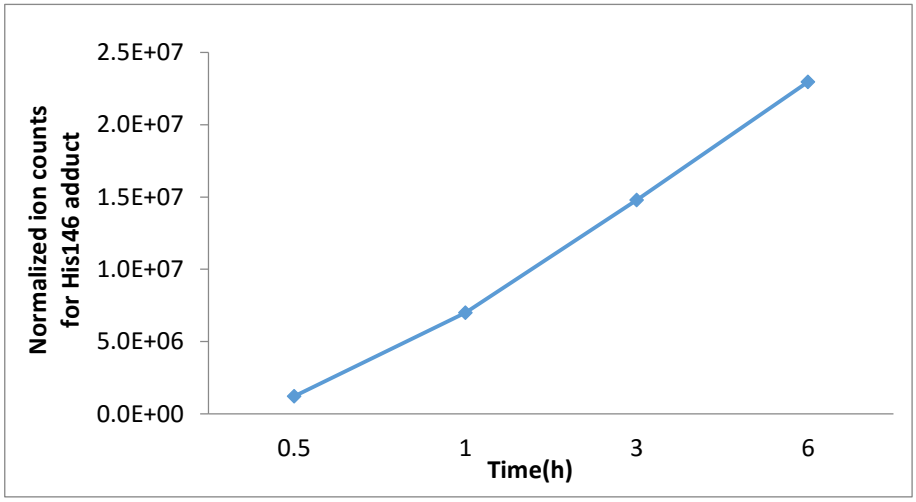

Cys34
His146
His338

C


Spectrum from XM HSA +CBZE 10,1 MRM-HR 111215.wiff (sample 1) - XM HSA +CBZE 10,1 MRM-HR 111215, Experiment 9, +TOF MS^2 of 717.7 (100 - 1800) from 70.401 min
145 RH(+CBZE)PYFYAPELLFFAK 159
(3+, 717.7, M*=2151.1
M=1899.0, Δm = 252.1)
+2, peptide
+3, peptide+252
+2,b7
+2,b7*
+2,y8
+2,b5
+2,b6
b6
b7
y8
y9
y10
b10
b11
b12
y13
950.0063
950.5081
964.5520
717.7052
718.0395
482.7809
432.7128
351.1816
468.2314
594.2772
701.3522
594.7793
864.4155
865.4171
965.5541
1198.6522
1274.6335
1387.7168
1534.7854
1605.8306
236.0716
210.0916
180.0812
253.0978
Intensity
Mass/Charge, Da
Spectrum from XM HSA +CBZE 10,1 MRM-HR 111215.wiff (sample 1) - XM HSA +CBZE 10,1 MRM-HR 111215, Experiment 9, +TOF MS^2 of 717.7 (100 - 1800) from 70.556 min
y9
y10
b7*
b6*
b9
b10
y11
b11
1035.5898
1187.5436
1198.6512
1036.5920
1161.5540
1199.6534
1116.5042
1117.5105
1162.5514
1160.5580
1088.5168
1246.6422
1274.6334
1275.6329
1345.7206
1387.7193
1388.7188
Intensity
Mass/Charge, Da
Spectrum from XM HSA +CBZE 10,1 MRM-HR 111215.wiff (sample 1) - XM HSA +CBZE 10,1 MRM-HR 111215, Experiment 9, +TOF MS^2 of 717.7 (100 - 1800) from 70.634 min
+2,b7
y8
+2,b6
+2,b5*
+2,b6*
+2,b7*
y3
+2,b4*
y4
482.7802
432.7128
468.2313
483.2826
512.2856
594.2769
365.2181
418.7155
433.2152
468.7329
403.6923
419.2174
454.2355
366.2201
389.6946
513.2881
518.2991
554.2824
555.2863
558.7569
581.2797
594.7796
572.3440
Intensity
Mass/Charge, Da
XIC from XM HSA +CBZE 10,1 MRM-HR 111215.wiff (sample 1) - XM HSA +CBZE 10,1 MRM-HR 111215, Experiment 1, +TOF MS (400 - 1500): 633.700 +/- 0.005 Da, Gaussian smoothed, Gaussian smoothed, Gaussian smoothed
XIC from XM HSA +CBZE 10,1 MRM-HR 111215.wiff (sample 1) - XM HSA +CBZE 10,1 MRM-HR 111215, Experiment 1, +TOF MS (400 - 1500): 717.700 +/- 0.005 Da, Gaussian smoothed, Gaussian smoothed, Gaussian smoothed
modified
unmodified
61.21
62.12
Intensity
Time, min

D


1.60E+08
1.20E+08
8.00E+07
4.00E+07
0.00E+00
Normalized ion counts for His146 adduct
0.1:1
1:1
5:1
Drug protein molar ratio


2.5E+07
2.0E+07
1.5E+07
1.0E+07
5.0E+06
0.0E+00
Normalized ion counts for His146 adduct
0.5
1
3
6
Time(h)

E

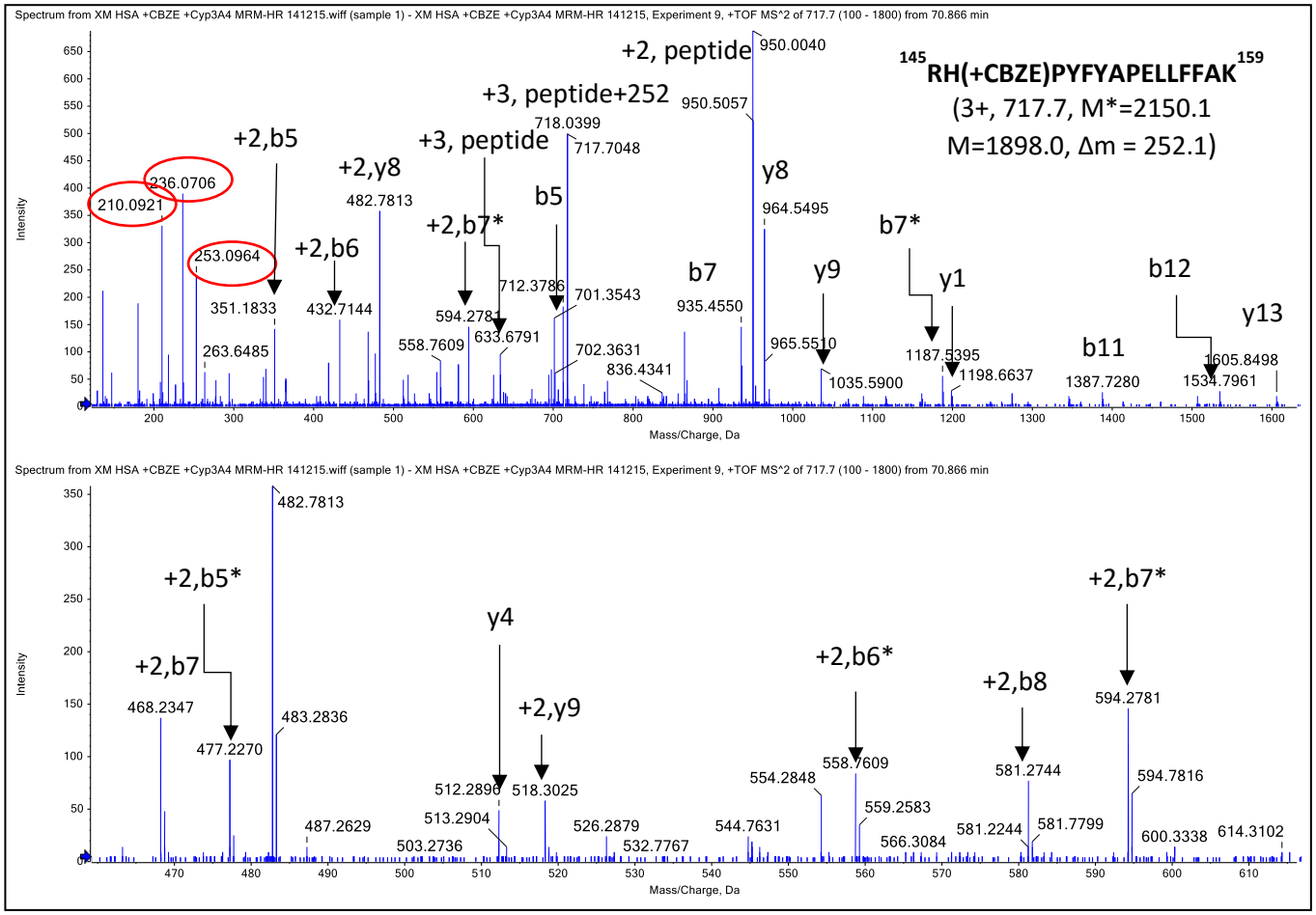
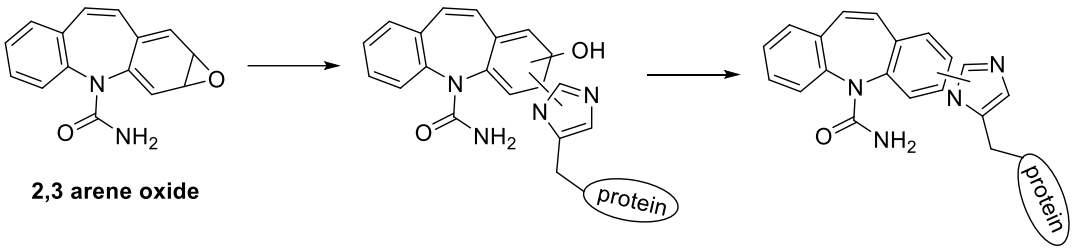

Spectrum from XM HSA +CBZE +Cyp3A4 MRM-HR 141215.wiff (sample 1) - XM HSA +CBZE +Cyp3A4 MRM-HR 141215, Experiment 9, +TOF MS^2 of 717.7 (100 - 1800) from 70.866 min
145 RH(+CBZE)PYFYAPELLFFAK 159
(3+, 717.7, M*=2150.1
M=1898.0, Δm = 252.1)
+2, peptide
950.0040
+3, peptide+252
950.5057
718.0399
717.7048
+3, peptide
+2,b5
+2,y8
482.7813
b5
y8
964.5495
+2,b7*
+2,b6
b7*
b7
y9
y1
b12
y13
b11
236.0706
210.0921
253.0964
351.1833
432.7144
712.3786
701.3543
594.278
633.6791
558.7609
263.6485
935.4550
965.5510
1187.5395
1605.8498
702.3631
836.4341
1035.5900
1198.6637
1387.7280
1534.7961
Intensity
Mass/Charge, Da
Spectrum from XM HSA +CBZE +Cyp3A4 MRM-HR 141215.wiff (sample 1) - XM HSA +CBZE +Cyp3A4 MRM-HR 141215, Experiment 9, +TOF MS^2 of 717.7 (100 - 1800) from 70.866 min
482.7813
+2,b5*
+2,b7
y4
+2,y9
+2,b6*
+2,b8
+2,b7*
468.2347
483.2836
477.2270
512.2896
518.3025
554.2848
558.7609
581.2744
594.2781
559.2583
594.7816
513.2904
487.2629
526.2879
544.7631
581.2244
581.7799
503.2736
532.7767
566.3084
600.3338
614.3102
Intensity
Mass/Charge, Da

Figure 4

A


2,3 arene oxide
protein
protein

B

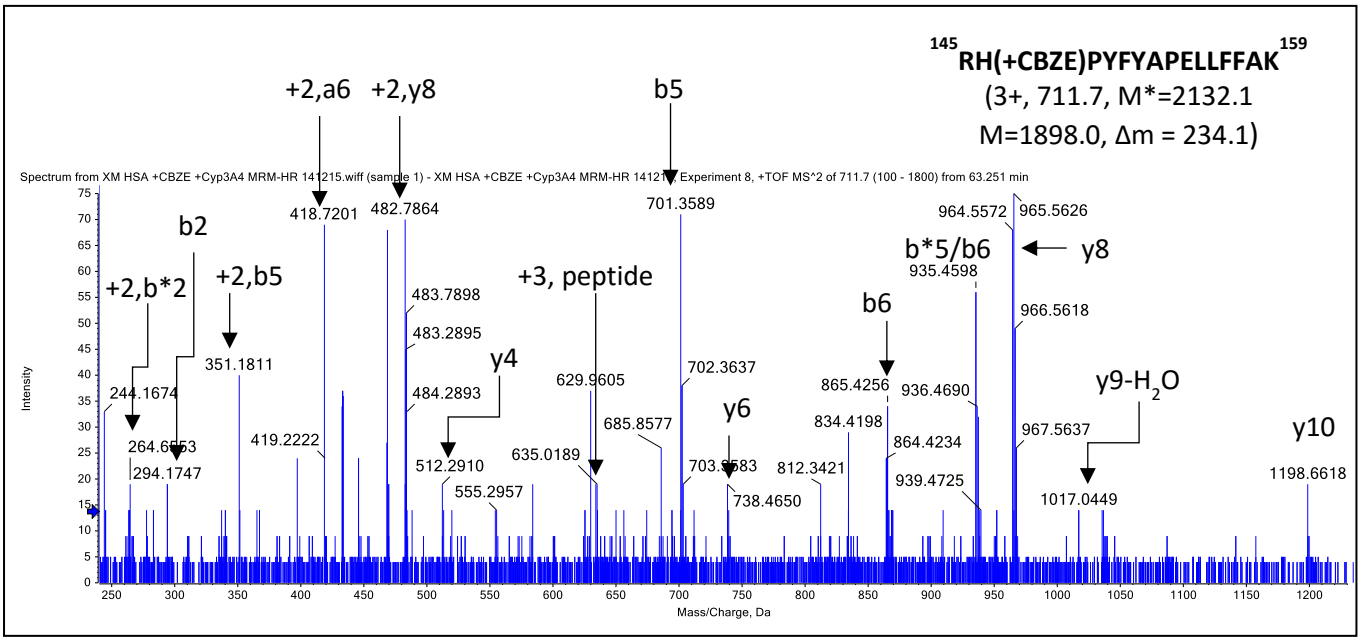
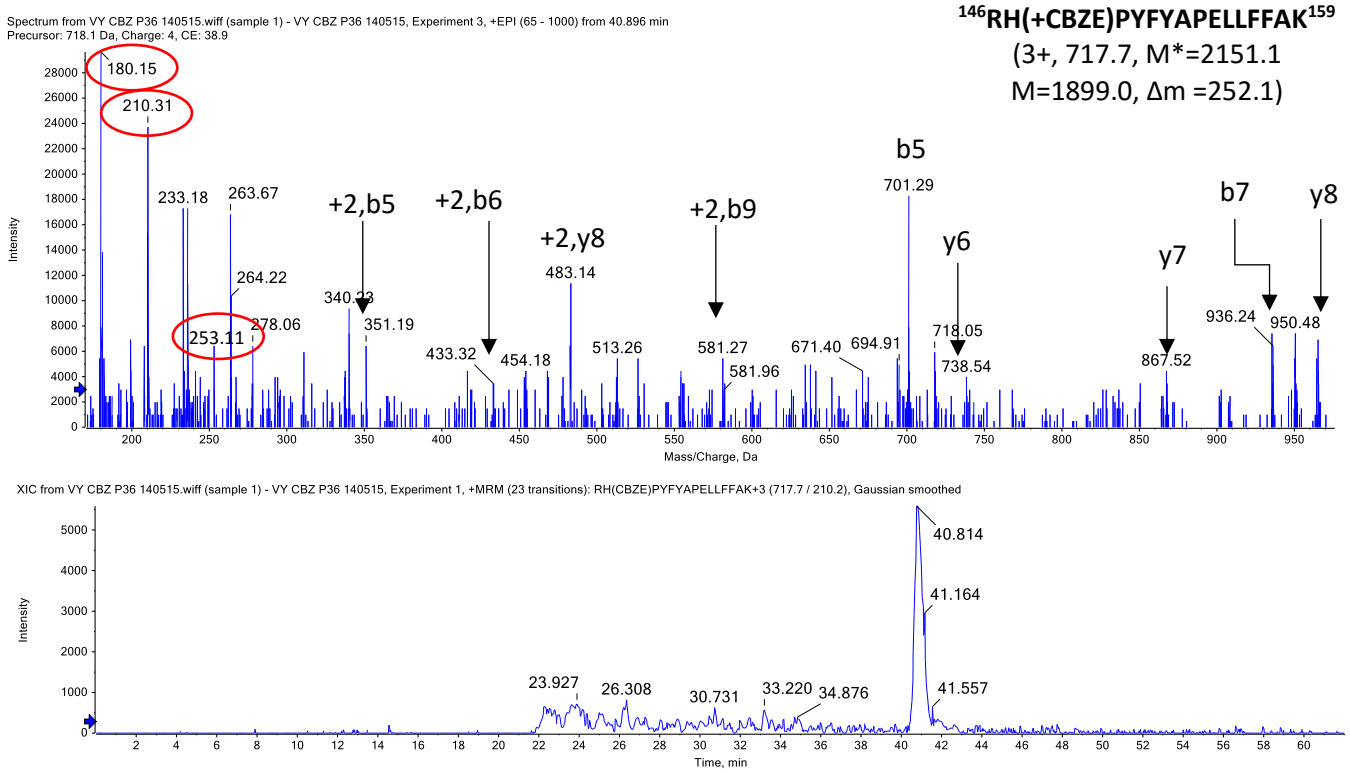

145 RH(+CBZE)PYFYAPELLFFAK 159
(3+, 711.7, M*=2132.1
M=1898.0, Δm = 234.1)
+2,a6
+2,y8
b5
b2
+2,b*2
+2,b5
+3, peptide
b6
b*5/b6
y8
y4
y6
y9-H2O
y10
Intensity
Mass/Charge, Da

Figure 5

(A)


Spectrum from VY CBZ P36 140515.wiff (sample 1) - VY CBZ P36 140515, Experiment 3, +EPI (65 - 1000) from 40.896 min
Precursor: 718.1 Da, Charge: 4, CE: 38.9
146RH(+CBZE)PYFYAPELLFFAK159
(3+, 717.7, M*=2151.1
M=1899.0, Δm =252.1)
Intensity
Mass/Charge, Da
180.15
210.31
233.18
263.67
264.22
253.11
278.06
340.23
351.19
433.32
454.18
483.14
513.26
581.27
581.96
671.40
694.91
701.29
718.05
738.54
867.52
936.24
950.48
+2,b5
+2,b6
+2,y8
+2,b9
b5
y6
y7
b7
y8
XIC from VY CBZ P36 140515.wiff (sample 1) - VY CBZ P36 140515, Experiment 1, +MRM (23 transitions): RH(CBZE)PYFYAPELLFFAK+3 (717.7 / 210.2), Gaussian smoothed
Intensity
Time, min
40.814
41.164
41.557
23.927
26.308
30.731
33.220
34.876

(B)

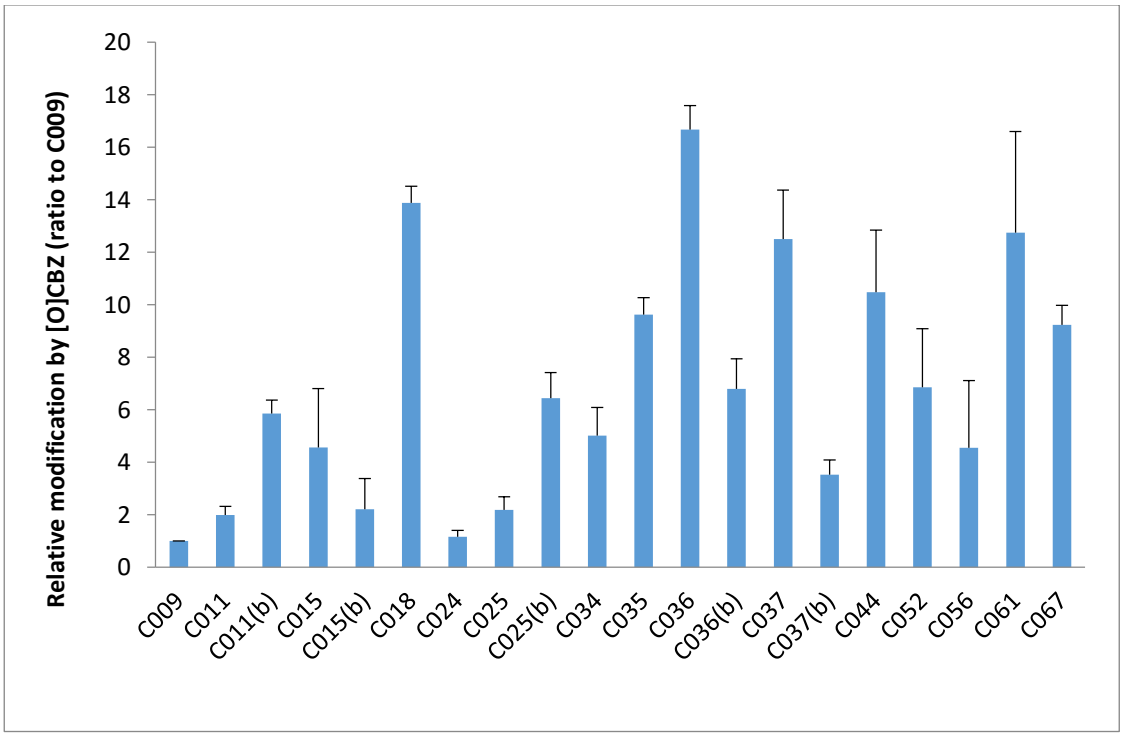
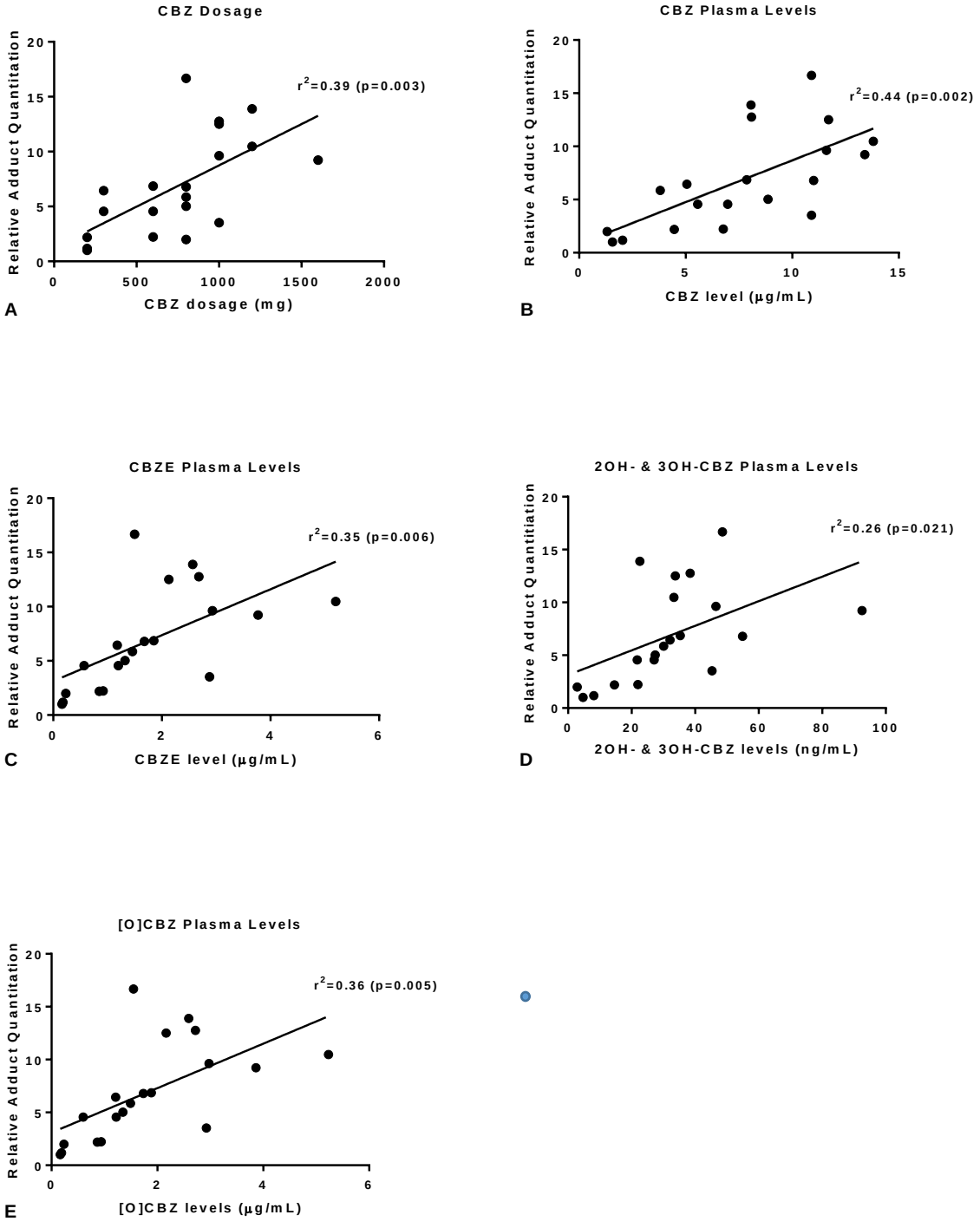

Relative modification by [O]CBZ (ratio to C009)
C009
C011
C011(b)
C015
C015(b)
C018
C024
C025
C025(b)
C034
C035
C036
C036(b)
C037
C037(b)
C044
C052
C056
C061
C067

Figure 6


CBZ Dosage
Relative Adduct Quantitation
r²=0.39 (p=0.003)
CBZ dosage (mg)
A
CBZ Plasma Levels
r²=0.44 (p=0.002)
CBZ level (µg/mL)
B
CBZE Plasma Levels
r²=0.35 (p=0.006)
CBZE level (µg/mL)
C
2OH- & 3OH-CBZ Plasma Levels
r²=0.26 (p=0.021)
2OH- & 3OH-CBZ levels (ng/mL)
D
[O]CBZ Plasma Levels
r²=0.36 (p=0.005)
[O]CBZ levels (µg/mL)
E